IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **Paul I. Freimuth, et al.**

Serial No.: **10/037,243** Group No. **1636**

Filed: **January 4, 2002** Examiner: **J. Leffers**

For: **Facilitating Protein Folding and Solubility by Use of Peptide Extensions**

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. §1.132

The Honorable Commissioner
of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Paul I Freimuth, declare and say:

1. I received a B.Sc. in Biology from the University of Connecticut in 1977 and an M.Sc. in 1983 and a Ph.D. in 1986 in Microbiology from Columbia University.

2. I was a postdoctoral fellow at Rockefeller University from 1986 to 1991.

3. I have been on the scientific staff of Brookhaven National Laboratory (BNL) since 1991. I am currently a Biochemist of the Biology Department at BNL and an adjunct professor of Microbiology at the State University of New York at Stony Brook.

4. I have published 30 peer-reviewed papers in the scientific literature. Much of my research since my postdoctoral studies has been directed toward the development of an

understanding of the interaction of viruses with their cellular receptors. These efforts have primarily been directed toward adenoviruses and the human coxsackie and adenovirus receptor protein. I have published approximately 15 papers and reviews related to this topic in the scientific literature.

5. I am an inventor of the above-referenced patent application.

6. **Remarks on Examiners comments, in particular, item 3.**

After a thorough review of the remarks by the Examiner and the literature cited by the Examiner, as a person knowledgeable in the field of molecular biology I wish to bring the following to the Examiner's attention in support of the patentabilty of the invention of the referenced application.

<u>Sizes of proteins that can be expressed by recombinant technology:</u>

It is well-established that various expression systems, i.e., type of cell and/or in vitro system, vectors, etc. have an influence on the likelihood or expectation of success when first contemplating accomplishing the expression of a new target protein. The teachings in the art are extensive and are readily available to persons of ordinary skill in the practice of recombinant gene expression. The trial and error aspects of accomplishing the expression of a new protein are greatly facilitated by the availability of the collections of reagents and systems offered by commercial organizations.

For example, it is well known that mRNAs have considerably shorter half-lives in bacterial cells than in eukaryotic cells. Thus, when a large protein (e.g. > 500 amino acids) is the target, it would be reasonable for the practitioner to first try a vector that could be used in both bacterial and eukaryotic cells, such as the dual vectors of Stratagene. Thus, through one cloning

systems would also be a likely starting point – and such dual vectors could easily be used in vitro.

Over the course of the multi-decade use of recombinant DNA technology to express proteins, proteins as small as peptide size (e.g. <100 amino acid residues) and as large as the catalytic subunit of DNA-PK (450 K daltons – i.e., >3,500 amino acid residues) have been prepared using recombinant DNA technology.

Conditions for expression – physiological:

It is extremely unlikely that one of ordinary skill in the art would contemplate trying to express proteins under non-physiological culture or in vitro conditions. Thus, to one of ordinary skill in the art, vectors for expressing recombinant genes would be used under physiological conditions and therefore the net negative charge of the peptide extensions of this invention are calculated with that assumption.

State of the art/Unpredictability of the art:

With respect to the Examiner's remarks that "there are vagaries as to protein production that often require considerable, costly and lengthy experimentation", I wish to state that it is very straightforward for persons of ordinary skill in the art to make and test many different vectors and expression systems to find the one that provides the simplest and optimal choice for expression of a new protein. It is no longer a costly or lengthy process, but is quite routine. Issues such as vector instability, vector loss, the stability/instability of the mRNAs are all issues that are commonplace and those of ordinary skill in the art are well-equipped to work with such variables.

With all of the research tools that are available to the molecular biology scientific community today, particularly in contrast to what was available 20 or more years ago, it is a

simple matter to purchase and use reagents and kits and systems that are designed, not for the most skilled, but for persons of ordinary skill in this art. Moving DNA sequences from one vector to another is simple and can be accomplished in less than one day's work – and when done in parallel, many modified vectors can be produced in a single day.

Applicability of "solubility partners" to a wide variety of proteins:

If a "solubility partner" was applicable to all proteins that are currently being made and will be made in the future using recombinant DNA techniques there would not be so many in common use today. It is an accepted state of the art that one may need to resort to trying several different "solubility partners" to find one that enhances solubility and folding of a particular target protein. In addition, it is also possible that none that are currently available are adequate and that one must then resort to trying alternate temperatures for expression, alternative cell types for expression, in vitro expression and also trying recovery from inclusion bodies. This trial and error process is quite routine.

Guidance in the specification:

When communicating the present invention to others of skill in the art of recombinant DNA technology and its use for the production of proteins, whether communicating by word of mouth or through presentation of the present specification, others have found that the invention to be well understood and practicable immediately upon learning the characteristics of the enhancing peptide extensions of this invention. In fact, when communicating with the scientists at Stratagene when they began development of the VariFlex™ system, there were certainly no questions about how to make and use the vectors of the present case. Their system now offers an incredibly accessible way in which even persons of minimal skill in the art could practice the invention. If there had been insufficient guidance in the teachings it is highly likely that their

studies would have ended in disappointment and that they would not have licensed and developed the VariFlex™ system. It is my belief that the ease with which the vectors were developed is testimony to the fact that the teachings of the specification are more than sufficient to enable any ordinarily skilled person to successful practice.

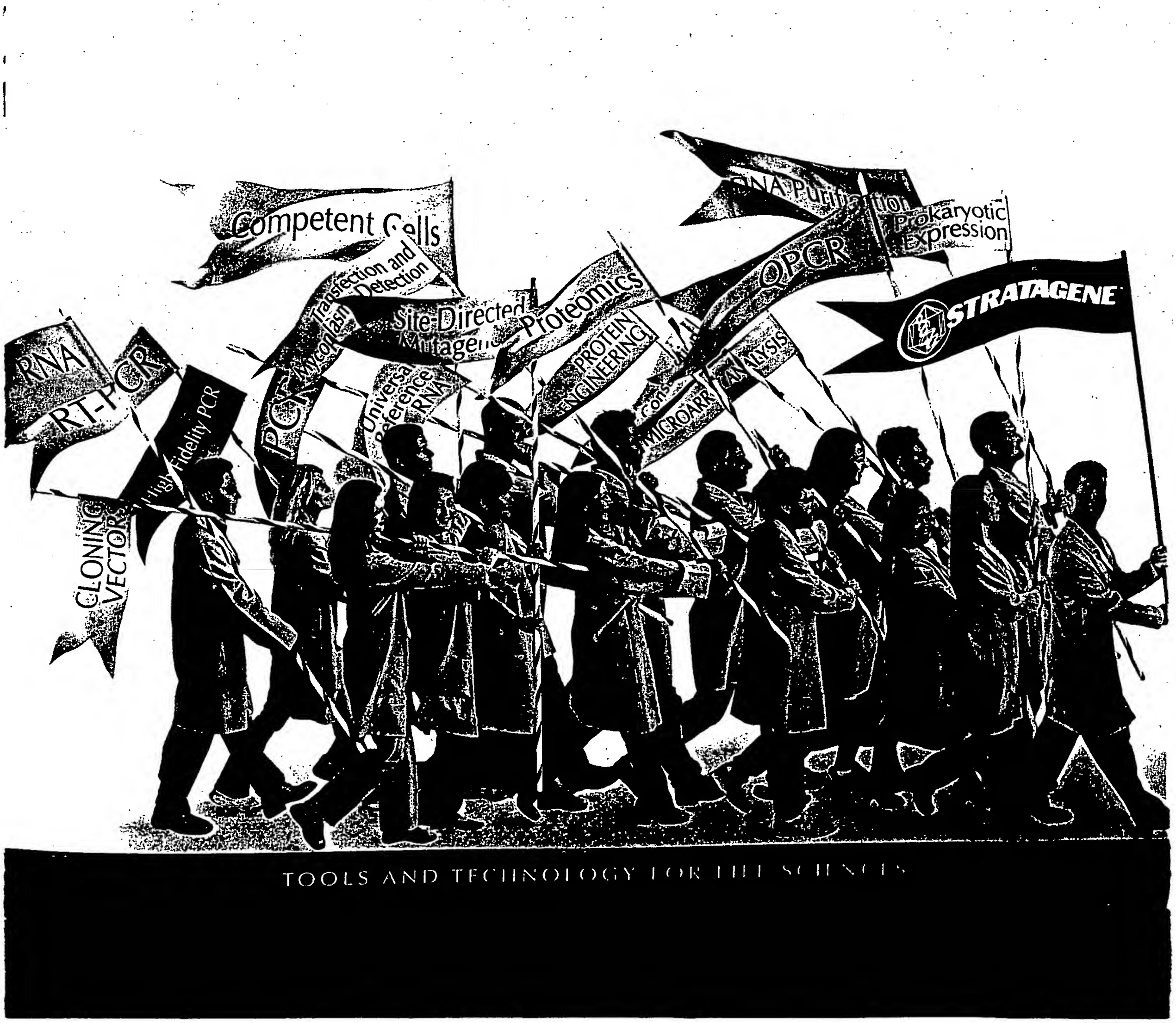
I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true. Further that these statements were made with the knowledge that willfully false statements, and the like, so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code, and that such willfully false statements may jeopardize the validity of patent that may issue from the referenced application.

Jan 20, 2005
Date

Paul Freimuth
Paul I. Freimuth, Ph. D.

STRATAGENE

2003/2004 CATALOG



PROTEIN EXPRESSION

MAMMALIAN EXPRESSION **84**

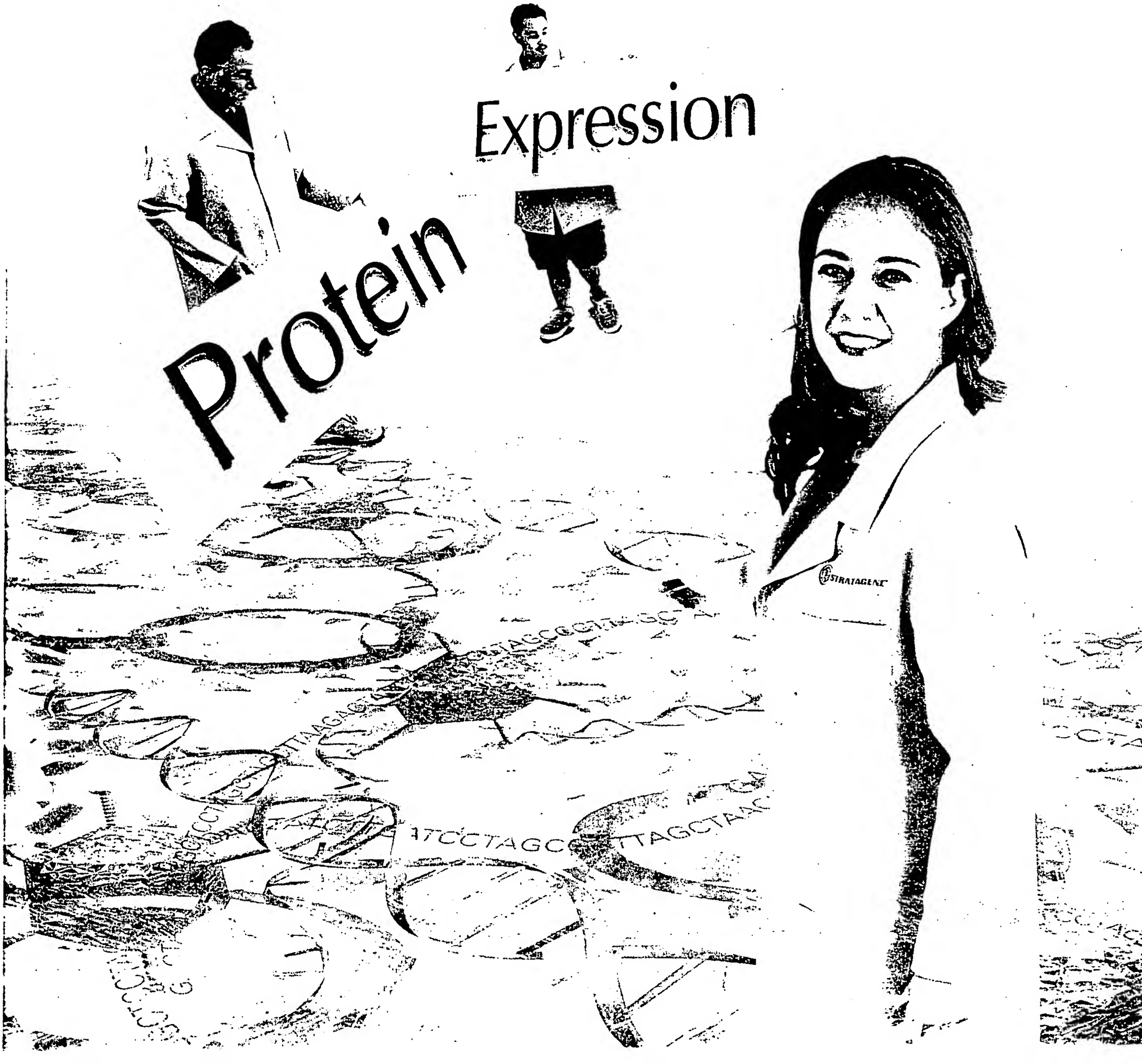
DUAL EXPRESSION **89**

INDUCIBLE MAMMALIAN EXPRESSION **91**

VIRAL EXPRESSION **94**

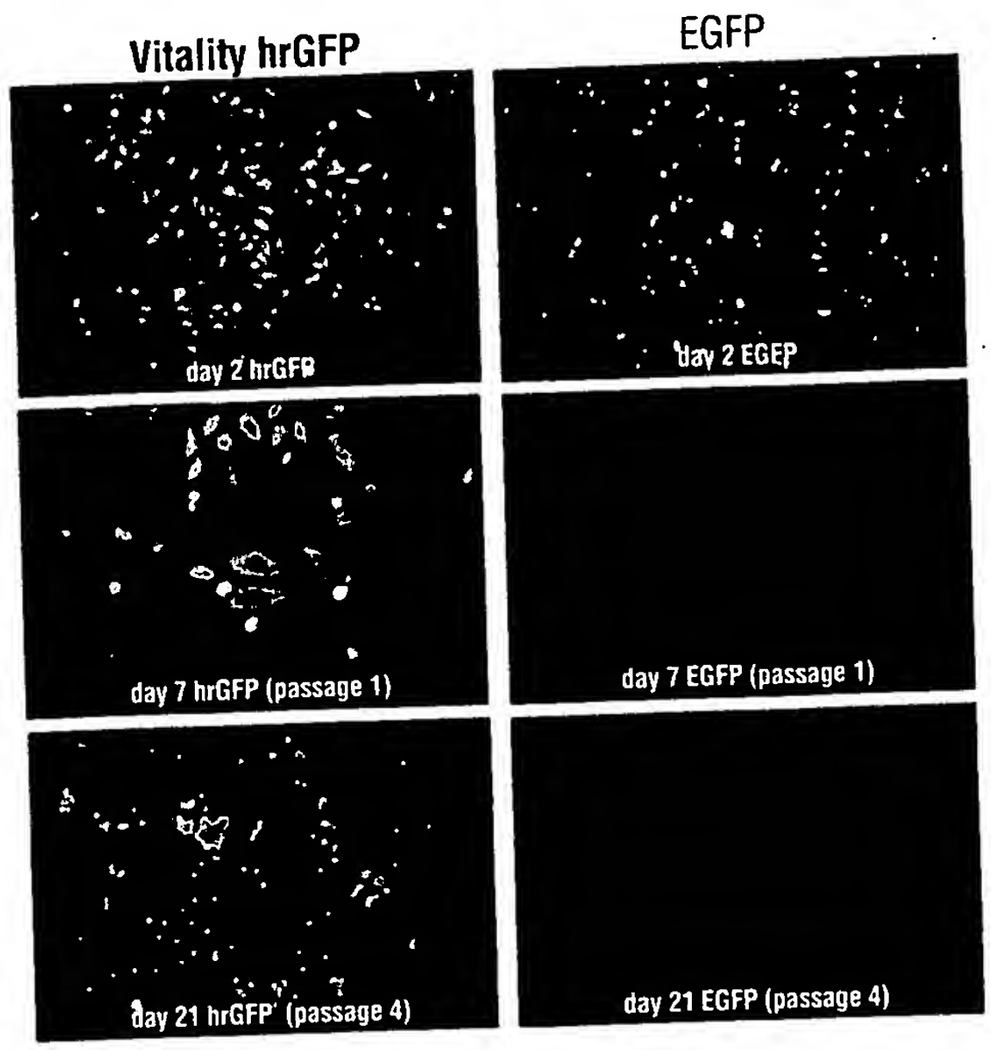
YEAST EXPRESSION **99**

PROKARYOTIC EXPRESSION **101**



MAMMALIAN EXPRESSION

Vitality™ hrGFP Mammalian Expression Vectors



Viability of Cells Expressing hrGFP vs. EGFP in Transduced HeLa Cells
Comparison of the relative viability of cells for high-level expression of each GFP over time and passage number. HeLa cells were transduced with retroviral vectors harboring either the hrGFP gene, or that for the red-shifted, humanized *Aequorea* protein (EGFP). Cells were transduced at an equivalent multiplicity of infection with one or the other virus, and then passaged for several weeks in the absence of any selection. At various times following transduction, the cell populations were photographed using a fluorescent microscope.

- New organism, new performance!
- High-level green fluorescence is easy to detect
- Low-toxicity reporter does not interfere with expression of your gene
- Stable hrGFP expression allows generation of stable cell lines
- Non-invasive detection in vivo using FACS or fluorescent microscope

New Organism, New Performance!

Stratagene has cloned and humanized a GFP from a different organism to create the new low-toxicity Vitality™ humanized *Renilla* GFP* (hrGFP). Users of the red-shifted, humanized *Aequorea* protein (EGFP) often complain of EGFP's unstable expression caused by high toxicity. As a result, the gene expression profile in both stable and transient experiments can be changed. Vitality hrGFP solves this problem.

Now Study Gene Expression in Healthy Cells

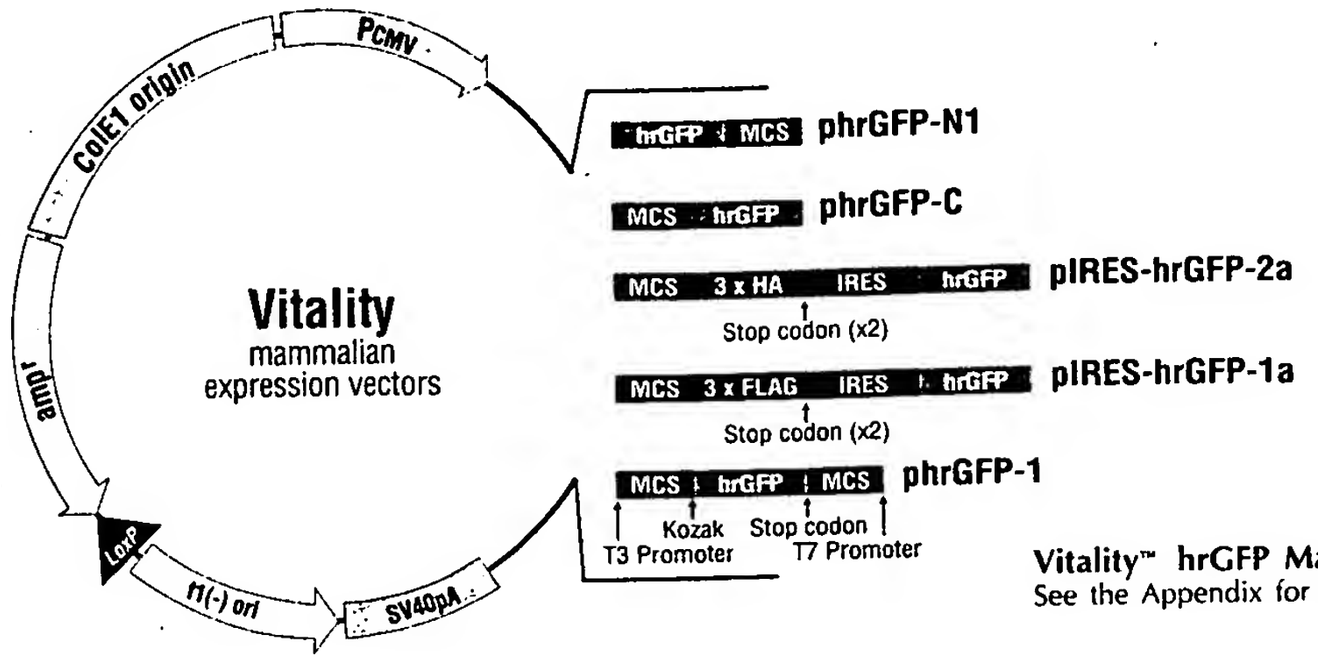
It is now possible to study gene expression without introducing undesirable artifacts into your experiment by using low toxicity Vitality hrGFP expression vectors. Our side-by-side experiments show that cells expressing hrGFP appear to be noticeably healthier than those expressing EGFP, both soon after transduction and over time. The EGFP-expressing cells either die or stop expressing the protein. In contrast, cells expressing hrGFP look healthy and show robust fluorescence for long periods of time with no loss of expression.

Versatile Expression Vectors

Vitality hrGFP mammalian expression vectors are available in a variety of vector formats for choosing the appropriate configuration for specific gene expression studies. The following table includes each Vitality hrGFP vector for monitoring gene expression and protein localization. All Vitality hrGFP vectors will fluoresce without an insert. This means you can transfect your cells and verify hrGFP expression before cloning.

APPLICATIONS
• Localize recombinant protein expression in vivo • Generate stable cell lines expressing hrGFP as a reporter • Use hrGFP to gauge transfection efficiency in vivo • Allows expression of native protein with small epitope tag • Evaluate different promoters or enhancers cloned upstream of the hrGFP reporter • Localize hrGFP to specific organelles to study organelle dynamics real-time in living cells • Easily insert different drug-resistance genes using *LoxP* site-specific recombination • Detect protein expression in vivo with fluorescent microscope or fluorescence-activated cell sorting (FACS)
SELECTION
• Mammalian: These vectors take advantage of *Cre*-mediated site-specific recombination to allow quick and efficient directional insertion of prefabricated modules. A variety of drug-resistant markers may be readily substituted into the core hrGFP vector bearing the gene of interest (hygromycin, puromycin or neomycin). • Bacterial: Ampicillin
REPLICATION ORIGINS
• F1 filamentous phage: Origin of replication allows recovery of single-stranded DNA • ColE1 origin: Plasmid origin of replication used in *E. coli* • SV40 origin: Mammalian origin of episomal replication requires expression of SV40 T antigen. SV40 promoter and origin is delivered into the vector by the puromycin and neomycin drug-resistance modules. The hygromycin drug-resistance module delivers its own endogenous promoter.
PROMOTERS and EXPRESSION
• T7 and T3 promoters allow RNA transcription, sequencing and PCR. • CMV immediate early promoter** drives mammalian expression. • SV40 polyadenylation sequence provides signals required for termination of mammalian transcription and translation.

* Patents pending. See license reference 37 on page 324.
** See license reference 4 on page 324.



Vitality™ hrGFP Mammalian Expression Vectors
See the Appendix for other Vitality hrGFP vector maps.

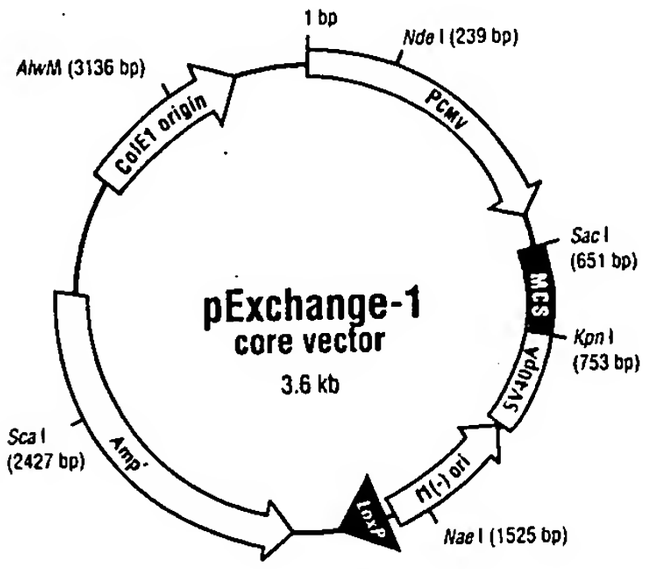
Vitality™ hrGFP Mammalian Expression Vectors

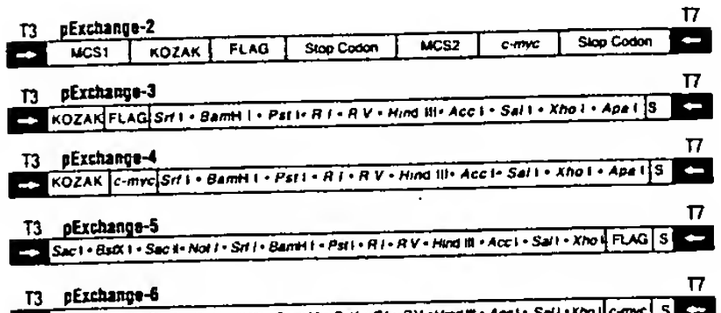
VECTOR	USE	CONTENTS	CATALOG #
Bicistronic Expression Vectors* *(LoxP site allows easy insertion of different drug-resistance genes using site-specific recombination)*			
pIRES-hrGFP-1a vector	• Express both the recombinant protein with FLAG fusion tag** and hrGFP from same mRNA transcript	20 µg	#240031
pIRES-hrGFP-2a vector	• Express both the recombinant protein with HA fusion tag and hrGFP from same mRNA transcript	20 µg	#240032
Protein Fusion Vectors *(LoxP site allows easy insertion of different drug-resistance genes using site-specific recombination)*			
phrGFP-C vector	• Express recombinant protein with hrGFP fused to the C-terminus	20 µg	#240035
phrGFP-N1 vector	• Express recombinant protein with hrGFP fused to the N-terminus	20 µg	#240036
Source of hrGFP Gene *(LoxP site allows easy insertion of different drug-resistance genes using site-specific recombination)*			
phrGFP-1 vector	• Easily transfer hrGFP gene to vector of choice using convenient restriction sites or PCR amplification	20 µg	#240059
Promoterless Vector *(LoxP site allows easy insertion of different drug-resistance genes using site-specific recombination)*			
phrGFP vector	• Evaluate various promoters or promoter/enhancer element combinations inserted into the MCS located upstream of hrGFP gene	20 µg	#240062
Subcellular Localization Vectors *(LoxP site allows easy insertion of different drug-resistance genes using site-specific recombination)*			
phrGFP-Mito vector	• Expression product targets hrGFP to the mitochondria	20 µg	#240042
phrGFP-Nuc vector	• Expression product targets hrGFP to the nucleus	20 µg	#240043
phrGFP-Golgi vector	• Expression product targets hrGFP to the golgi	20 µg	#240044
phrGFP-Perox vector	• Expression product targets hrGFP to the peroxisomes	20 µg	#240063
Retroviral Reporter Vectors			
pFB-hrGFP vector	• Use to determine retroviral transduction efficiency where expression cannot be determined directly	10 µg	#240027
pFB-hrGFP retroviral supernatants	• Same as above but eliminates need to generate infectious virus particles • Titer: 2.7 x 10^7	1.8 ml	#972002
Related Products			
pExchange module EC-Hyg	• Hygromycin drug-resistance module contains *LoxP* site allowing introduction of module into *LoxP* site of Vitality hrGFP mammalian expression vector	1 µg (10 rxn)	#211181
pExchange module EC-Puro	• Puromycin drug-resistance module contains *LoxP* site allowing introduction of module into *LoxP* site of Vitality hrGFP mammalian expression vector	1 µg (10 rxn)	#211182
pExchange module EC-Neo	• Neomycin drug-resistance module contains *LoxP* site allowing introduction of module into *LoxP* site of Vitality hrGFP mammalian expression vector	1 µg (10 rxn)	#211183
Cre Recombinase	• Mediates recombination of *LoxP* sites • Includes 10X *Cre* recombinase buffer	150 U (30 rxn)	#600270
Anti-FLAG® M2 Antibody***	• Use to label FLAG fusion tag for Western analysis	200 µg	#200471
		1 mg	#200472

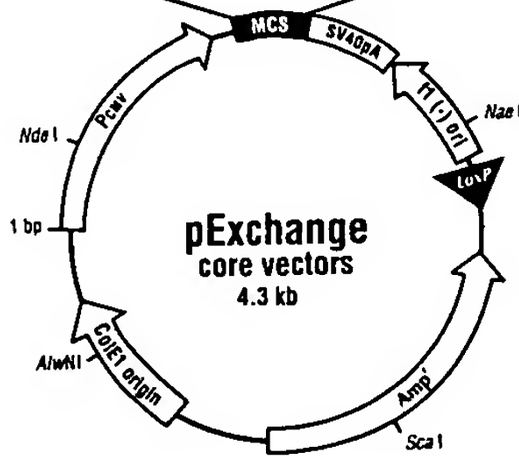
* See license reference 15 on page 324.
** See license reference 18 on page 324.
*** See license reference 8 on page 324.

The Exchanger™ System

- Clone once and quickly obtain your gene of interest in 3 different constructs with different drug-resistance genes
- Unparalleled convenience
- Quick and efficient insertion of drug-resistance modules with > 90% efficiency
- Entire process takes only 45 minutes
- CMV promoter* for expression in wide variety of eukaryotic cell types







APPLICATIONS
• Generate 3 different constructs harboring gene of interest with 3 different drug-resistance genes • Express epitope-tagged fusion proteins in mammalian cells • Screening by functional analysis, antibody or nucleic acid probes • Epitope Tagging (pExchange-2 through 6 vectors): Western blotting, immunofluorescence microscopy, immunoprecipitation, pull-down experiments and affinity chromatography
SELECTION
• Bacterial selection: Ampicillin • Mammalian selection: *Cre*-mediated site-specific recombination allows quick and efficient directional insertion of prefabricated modules. A variety of drug-resistant markers may be readily subsituted into the core vector harboring the gene of interest.
REPLICATION ORIGINS
• F1 filamentous phage: Origin of replication allows recovery of single-stranded DNA • ColE1 origin: Plasmid origin of replication used in *E. coli* • SV40 origin: Mammalian origin of episomal replication requires expression of SV40 T antigen. SV40 promoter and origin is delivered into the vector by the puromycin and neomycin drug-resistance modules. The hygromycin drug-resistance module delivers its own endogenous promoter.
PROMOTERS and EXPRESSION
• T7 and T3 promoters allow RNA transcription, sequencing and PCR. • CMV immediate early promoter drives mammalian expression. • SV40 polyadenylation sequence provides signals for termination of mammalian transcription and translation.

Convenient Exchange of Selectable Markers

The Exchanger™ system is a convenient way to create multiple expression constructs with different drug-resistance genes. After your gene of interest is cloned, linearized drug-resistance modules are inserted into the pExchange core vectors through site-specific recombination. This is accomplished without moving your gene of interest, eliminating the need to repeat sequencing and expression verification. In vitro insertion is by *Cre*-mediated recombination of *LoxP* sites, and recombination products are selected via drug resistance. The entire procedure consists of a 30-minute recombination reaction, a 15-minute heat inactivation, and transformation of suitable hosts with the recombination reaction. Desired reaction products are obtained with >90% efficiency!

Drug-Resistance Modules

The Exchanger system allows the introduction of a desired eukaryotic *LoxP* site for insertion of the hygromycin-, puromycin-, or neomycin-resistance module into the *LoxP* site of the core vector. The hygromycin- and puromycin-resistance modules also have a chloramphenicol-resistance marker for selection.

* See license reference 4 on page 324.

The Exchanger™ System

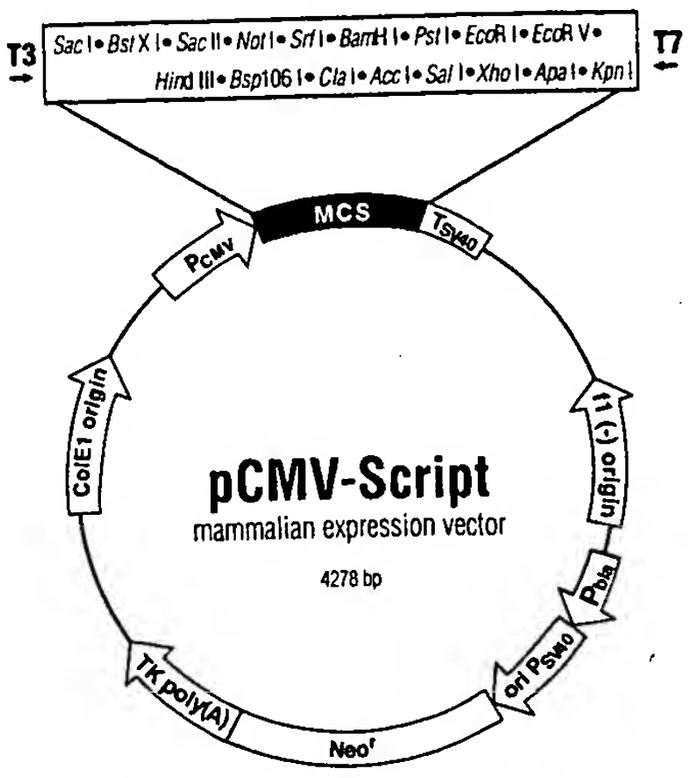
	CONTENTS	FEATURES	CATALOG #
pExchange Core Vectors			
pExchange-1 core vector	20 µg vector	• High-level expression • High-fidelity PCR cloning	#211176
pExchange-2 core vector		• N-, C-terminal or internal tagging with FLAG®* and c-myc epitopes	#211177
pExchange-3A, 3B, 3C core vectors	20 µg each vector, Corresponding pExchange-Luc control	• Contain N-terminal FLAG tag available in all 3 reading frames	#240001
pExchange-4A, 4B, 4C core vectors		• Contain an N-terminal c-myc tag available in all 3 reading frames	#240002
pExchange-5A, 5B, 5C core vectors		• Contain a C-terminal FLAG tag available in all 3 reading frames	#240003
pExchange-6A, 6B, 6C core vectors		• Contain a C-terminal c-myc tag available in all 3 reading frames	#240004
pExchange Drug-Resistance Modules (contain *LoxP* site which allows introduction of module into *LoxP* site of pExchange core vectors)			
pExchange module EC-Hyg	1 µg (10 rxn)	• Hygromycin drug-resistance module	#211181
pExchange module EC-Puro		• Puromycin drug-resistance module	#211182
pExchange module EC-Neo		• Neomycin drug-resistance module	#211183
The Exchanger™ System			
Exchanger system with pExchange-1 vector	20 µg pExchange-1 core vector, pExchange-1-Luc control, 150U *Cre* recombinase and buffer, pExchange modules: EC-Hyg, EC-Puro, EC-Neo, XL1-Blue supercompetent cells		#211184
Exchanger system with pExchange-2 vector	20 µg pExchange-2 core vector, pExchange-2-Luc control, 150U *Cre* recombinase and buffer, pExchange modules: EC-Hyg, EC-Puro, EC-Neo, XL1-Blue supercompetent cells		#211185
Anti-FLAG® M2 Antibody**	200 µg		#200471
	1 mg		#200472
***Cre* Recombinase**	150 U (30 rxn)	• Mediates recombination of *LoxP* sites, includes *Cre* 10X buffer	#600270

* See license reference 18 on page 324. ** See license reference 8 on page 324.

pCMV-Script® Vector

- CMV promoter* for high-level protein expression in mammalian cells
- Large multiple cloning site contains 15 unique sites
- Clone PCR products generated with any PCR enzyme

APPLICATIONS
• Protein expression in mammalian cells • Screening by functional assays, antibody or nucleic acid probes • PCR cloning
SELECTION
• Kanamycin resistance in bacteria, G418 resistance in mammalian cells
REPLICATION ORIGINS
• f1 filamentous phage: Origin of replication allows recovery of single-stranded DNA • ColE1 origin: Plasmid origin of replication used in *E. coli* • SV40 origin: Mammalian origin of episomal replication requires expression of SV40 T antigen
PROMOTERS and EXPRESSION
• T7 and T3 promoters allow RNA transcription, sequencing and PCR. • Mammalian expression is driven by the CMV immediate early promoter. • The SV40 polyadenylation sequence provides signals required for termination of mammalian transcription and translation.



High-Level Mammalian Expression

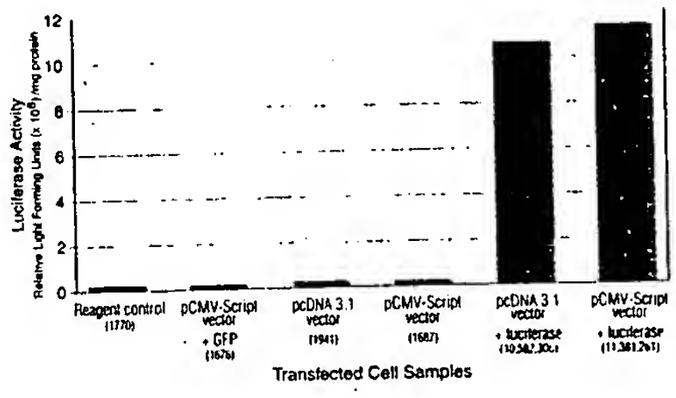
The pCMV-Script® vector is designed for high-level expression in mammalian cells. Expression is driven by the human cytomegalovirus (CMV) immediate early promoter. The powerful CMV promoter and SV40 polyadenylation site allow high-level constitutive expression of cloned inserts in a wide variety of cell lines. Stable clone selection is made possible with G418 by the presence of the neomycin-resistance gene.

Small Vector Size

The pCMV-Script vector is only 4.3 kb, allowing easy cloning and vector manipulations. This small size is due to the single antibiotic selection marker used by both prokaryotic and mammalian cells. The neomycin-resistance gene provides stable selection in mammalian cells with G418 driven by the SV40 promoter and kanamycin selection in *E. coli* cells driven by the β-lactamase promoter.

PCR Cloning

The pCMV-Script PCR cloning kit** is for high-efficiency PCR cloning. This kit provides all necessary reagents to clone PCR products into the pCMV-Script vector. > 70% efficiency is guaranteed with the test insert. See page 29 for more information on the pCMV-Script PCR cloning kit.



Luciferase Expression in the pCMV-Script® Vector
Constructs were transfected using lipid transfection methods into CHO cells. The cells were harvested, lysed and assayed for luciferase activity.

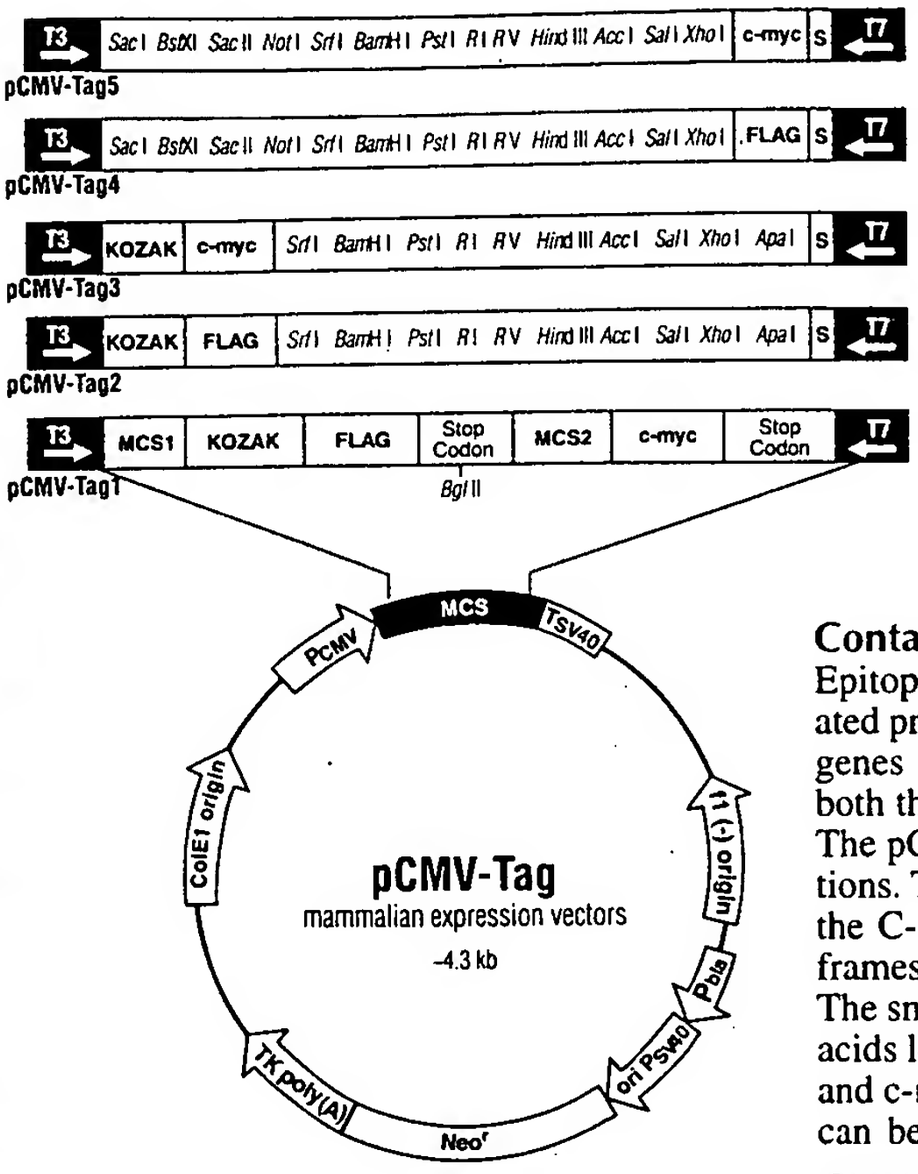
* See license reference 4 on page 324.
** See license reference 29 on page 324.

pCMV-Script® Vector Kits

AMOUNT	CONTENTS	FEATURES	CATALOG #
pCMV-Script® Vector Kit			
20 µg	pCMV-Script vector (supercoiled) XL1-Blue MRF' host strain	• Undigested	#212220
pCMV-Script® XR Predigested Vector			
55 rxn	pCMV-Script XR vector (digested with *Xho* I and *Eco*R I) Test insert	• Digested with *Xho* I and *Eco*R I for directional cloning	#212224
pCMV-Script® XR Library Construction Kit			
5 rxn	pCMV-Script XR vector (digested with *Xho* I and *Eco*R I) cDNA Synthesis Kit, XL10-Gold® ultracompetent cells	• Reagents for 5 cDNA synthesis reactions • For directional cloning	#200465
pCMV-Script® PCR Cloning Kit			
25 rxn	pCMV-Script vector Polishing reagents, *Srf* I restriction enzyme and ligation reagents, XL10-Gold ultracompetent cells StrataPrep® PCR purification kit, Controls	• Includes all necessary reagents to clone PCR products into the pCMV-Script vector	#211199

pCMV-Tag Vectors

- Provide both c-myc and FLAG® epitope-tagging options
- Eliminates the need to create gene-specific antibodies
- Features small tags that won't interfere with function of tagged protein



APPLICATIONS
• Express epitope-tagged fusion proteins in mammalian cells • Screening by functional assays, antibody or nucleic acid probes
SELECTION
• Bacterial selection: Kanamycin, Mammalian selection: G418
REPLICATION ORIGINS
• F1 filamentous phage: Origin of replication allows recovery of single-stranded DNA • ColE1 origin: Plasmid origin of replication used in *E. coli* • SV40 origin: Mammalian origin of episomal replication requires expression of SV40 T antigen
PROMOTERS and EXPRESSION
• T7 and T3 promoters allow RNA transcription, sequencing and PCR • CMV immediate early promoter drives mammalian expression • SV40 polyadenylation sequence provides signals required for termination of mammalian transcription and translation

Contain FLAG® and/or c-myc Epitope Tags

Epitope tags provide a method to localize gene products in living cells, identify associated proteins, track the movement of tagged proteins within a cell, and characterize new genes without creating protein-specific antibodies. The pCMV-Tag1 vector contains both the synthetic FLAG epitope* and the c-myc epitope from the human c-myc gene. The pCMV-Tag1 vector allows production of fusion proteins in a variety of conformations. The pCMV-Tag2-5 vectors allow either c-myc or FLAG epitope tagging at either the C- or N-terminus. Each pCMV-Tag2-5 vector is supplied with all three reading frames for easy cloning and expression.

The small size of the FLAG (eight amino acids long) and c-myc epitope tags (ten amino acids long) decreases the possibility of interference with the tagged protein. The FLAG and c-myc epitopes are recognized by the anti-c-myc and anti-FLAG antibodies, which can be used to characterize the target protein.

CMV Promoter for Expression in Mammalian Cells

The pCMV-Tag vectors allow for high-level expression of tagged proteins in mammalian cells, driven by the human cytomegalovirus (CMV) immediate early promoter.** The CMV promoter provides constitutive expression of cloned genes in a wide variety of cell lines. The translational start sequence used in the pCMV-Tag1, pCMV-Tag2 and pCMV-Tag3 vectors is a 10-base Kozak consensus sequence of GCC(A/G)CCATGG. pCMV-Tag4 and pCMV-Tag5 vectors do not contain a translation start sequence. Stable clone selection with G418 is possible due to the neomycin-resistance gene.

Small Vector Size

pCMV-Tag vectors are only 4.3 kb, allowing easy cloning and vector manipulations. This small size is due to the single antibiotic selection marker used by both prokaryotic and mammalian cells. The neomycin-resistance gene provides stable selection in mammalian cells with G418, driven by the SV40 promoter and kanamycin selection in *E. coli* cells driven by the *bla* (ß-lactamase) promoter.

* See license reference 18 on page 324.
** See license reference 4 on page 324.

pCMV-Tag Vectors

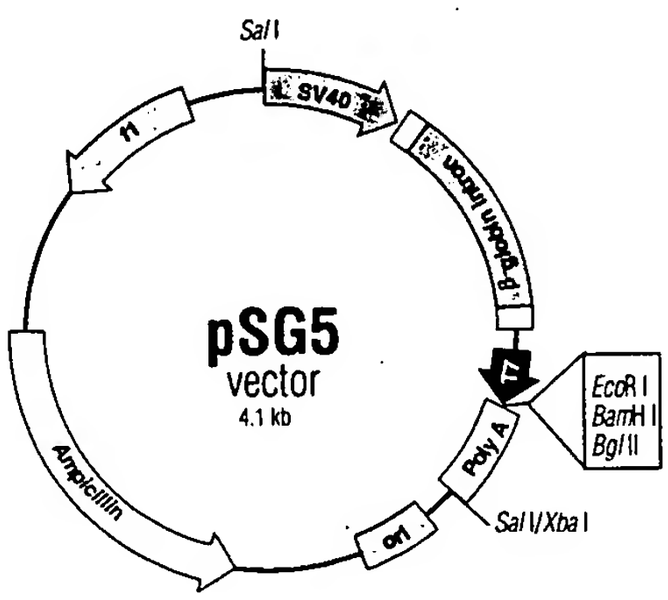
	CONTENTS	FEATURES	CATALOG #
pCMV-Tag1 vector	20 µg pCMV-Tag1 vector 10 µg pCMV-Tag1 control vector	• Both c-myc and FLAG tags	#211170
pCMV-Tag2 vector	20 µg each reading frame 10 µg pCMV-Tag2 control vector	• N-terminal FLAG tag	#211172
pCMV-Tag3 vector	20 µg each reading frame 10 µg pCMV-Tag control vector	• N-terminal c-myc tag	#211173
pCMV-Tag4 vector	20 µg each reading frame 10 µg pCMV-Tag control vector	• C-terminal FLAG tag	#211174
pCMV-Tag5 vector	20 µg each reading frame 10 µg pCMV-Tag control vector	• C-terminal c-myc tag	#211175
Anti-FLAG® M2 Antibody*			
	200 µg	• Recognizes FLAG epitope • Use to purify and characterize the target protein	#200471
	1 mg		#200472

* See license reference 8 on page 324.

pSG5 Vector

- 4.1-kb eukaryotic expression vector
- High copy number yields large quantities of double-stranded DNA

APPLICATIONS
• In vivo or in vitro expression • In vivo expression accomplished via transient expression of variety of cells • Highest level of expression produced in cells also expressing T antigen
SELECTION
• Bacterial selection: Ampicillin
REPLICATION ORIGINS
• F1 filamentous phage origin allows recovery of single-stranded DNA
PROMOTERS and EXPRESSION
• T7 bacteriophage promoter facilitates in vitro transcription of cloned inserts • Early SV40 promoter facilitates in vivo expression • Polyadenylation signal greatly increases level of expression
TRANSCRIPTION
• Intron II of rabbit ß-globin gene for splicing of expressed transcript



pSG5 Vector Kit

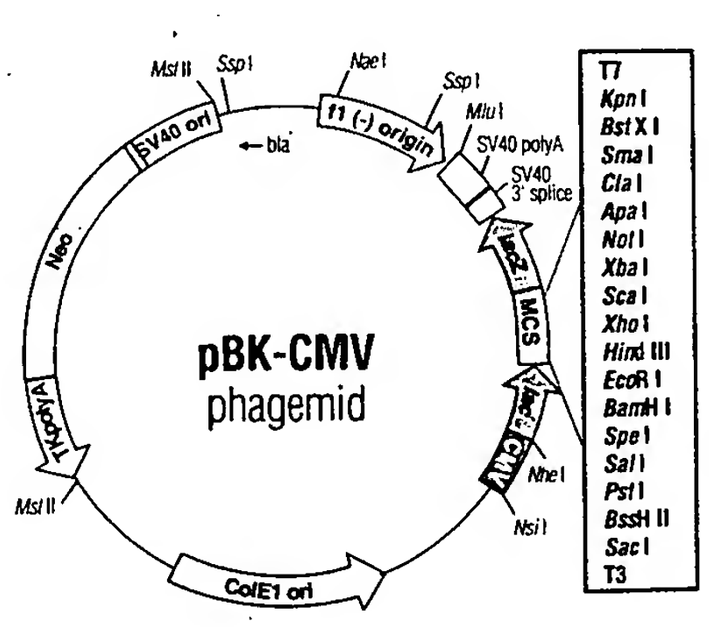
AMOUNT	CONTENTS	CATALOG #
20 µg	pSG5 vector Host strain: AG1	#216201

DUAL EXPRESSION

pBK-CMV Phagemid Vector

- Allows both prokaryotic and eukaryotic expression
- Rescued from ZAP Express® lambda vector

APPLICATIONS
• High-resolution restriction mapping • Creation of serial exo/mung deletions • f1 origin in the minus orientation allows rescue of single-stranded DNA • Double- and single-stranded sequencing
SELECTION
• Kanamycin resistance in bacteria and G418 resistance in eukaryotic cells
SCREENING
• Blue/white color screening in *E. coli*
CLONING SITES
• Polylinker with 17 unique sites
TRANSCRIPTION
• In vitro RNA transcription with T3 or T7 RNA polymerase
PROMOTERS and EXPRESSION
• Expression of fusion proteins/screening with antibody probes

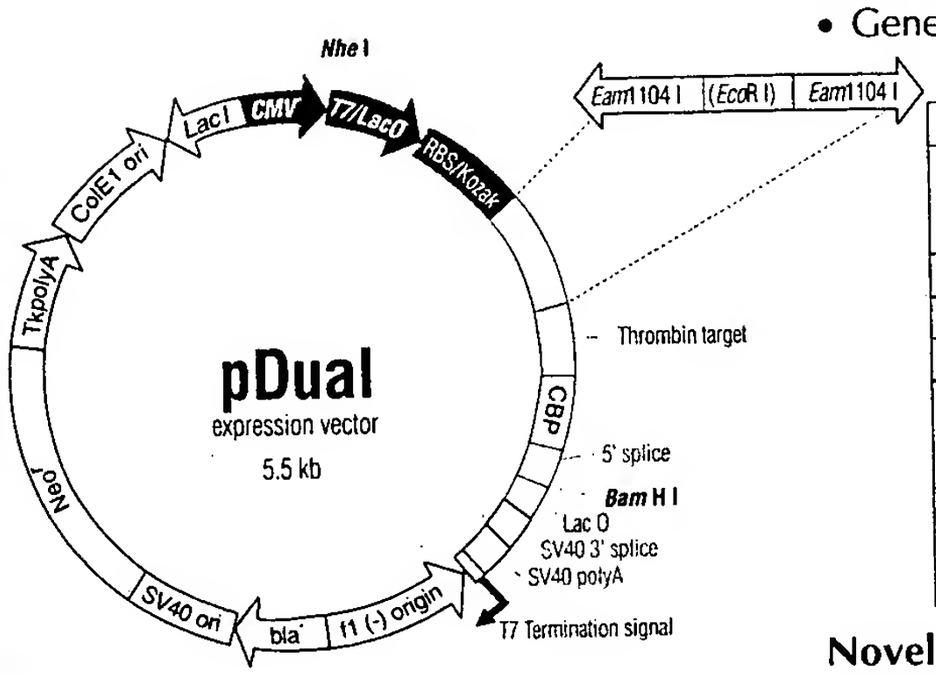


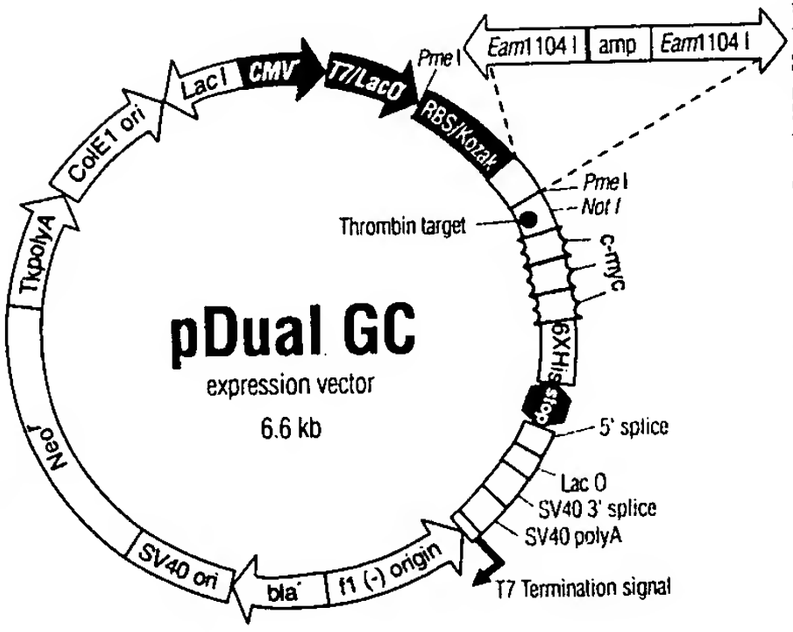
pBK-CMV Phagemid Vector Kit

AMOUNT	CONTENTS	FEATURES	CATALOG #
20 µg	pBK-CMV phagemid vector Host strain: XL1-Blue MRF' Helper phage: R408	• Allows both prokaryotic and eukaryotic expression	#212209

pDual® Expression System

- High-level protein expression in bacterial or mammalian systems
- Saves time by eliminating the need to subclone
- Express protein as a native or tagged fusion protein
- CBP- tagged protein for simple detection and affinity purification
- Clone seamlessly without compatible restriction sites
- *Eam*1104 I restriction sites are compatible with the Seamless® cloning technique
- Eliminates extraneous nucleotides for seamless cloning
- Generates nonpalindromic sticky ends for directional cloning





APPLICATIONS
• For both bacterial and mammalian cell expression; eliminates subcloning steps • Localize expression of protein of interest using epitope tag • Purify expressed protein using either Calmodulin Binding Protein or HIS6
SELECTION
• Bacterial selection: Kanamycin, Mammalian selection: G418
REPLICATION ORIGINS
• F1 filamentous phage: Origin of replication allows recovery of single-stranded DNA • ColE1 origin: Plasmid origin of replication used in *E. coli* • SV40 origin: Mammalian origin of episomal replication requires expression of SV40
PROMOTERS and EXPRESSION
• CMV immediate early promoter drives mammalian expression • Hybrid T7/*lacO* promoter for inducible bacteria expression

Novel Dual-Expression Vector

The pDual® expression vector* offers high-level expression of heterologous genes in both eukaryotic and prokaryotic systems. It contains the promoter and enhancer region of the human cytomegalovirus (CMV)** immediate early gene for constitutive expression in mammalian cells, expression in bacteria is regulated by a hybrid T7/*lacO* promoter,*** and carries the *lacI*^{q} repressor gene which allows for tight repression. In the presence of IPTG, protein is expressed in bacteria that contain the T7 RNA polymerase gene (e.g. BL21(DE3), BL21pLysS). In both bacterial and mammalian cells, the dominant selectable marker is the neomycin phosphotransferase gene under the control of both the β-lactamase and SV40 promoters, respectively. A special feature of the pDual vector is the tandemly arranged bacterial Shine-Dalgarno[1] and mammalian Kozak[2] consensus sequence. Each ribosome binding site is positioned at its optimal distance from the initiation codon of the target gene, for translation of mRNA generated in either system.

c-myc Epitope and HIS6 Tags for Easy Detection and Purification

The new pDual GC vector offers a C-terminal tag with three copies of the human c-myc epitope tag (one copy is EQKLISEEDL) and a single copy of the HIS6 purification tag. The c-myc epitope and HIS6 purification tags, expressed on the C-terminus of the protein, can be used for easy detection and purification of the fusion protein from mammalian and bacterial cell lysates, respectively. Use of the c-myc epitope and HIS6 purification tags eliminates the need to generate an antibody and optimized protein purification protocol for every target gene product.

REFERENCES
1. Shine, J. and Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. USA* 71: 1342-1346.
2. Kozak, M. (1986) *Cell* 44: 283-292.
3. Padgett, K.A. and Sorge, J.A. (1998) *Strategies* 10: 97-99.

* Patents pending
** See license reference 4 on page 324.
*** See license reference 6 on page 324.

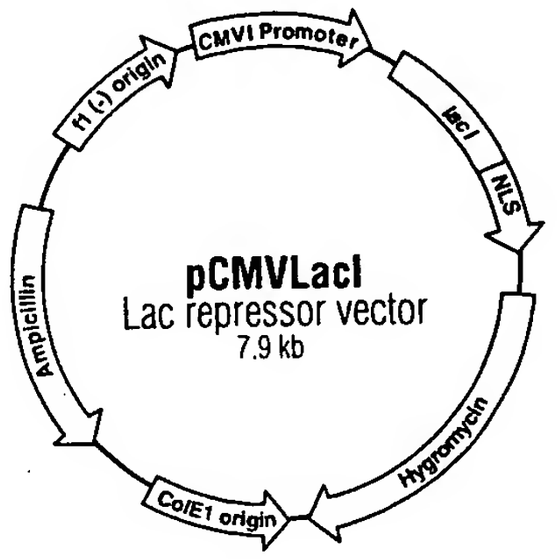
pDual® Expression System

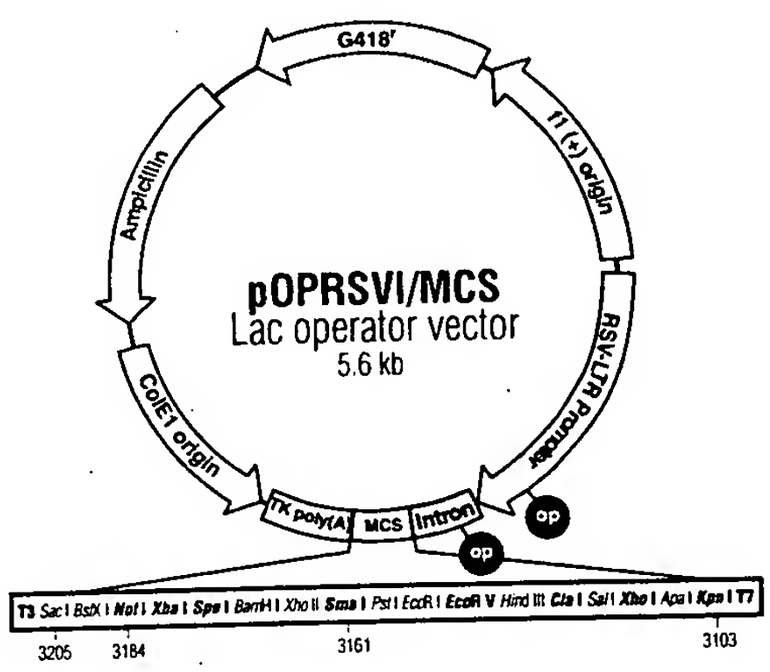
AMOUNT	CONTENTS	DESCRIPTION	CATALOG #
pDual GC Expression Vector			
20 µg	1µg/µl in TE buffer	• Uncut vector with c-myc epitope and HIS6 purification tag	#214503
pDual Expression Vector			
20 µg	pDual vector, undigested XL1-Blue host strain	• Uncut vector with CBP tag	#214501
100 µg			#214502
pDual Expression and Cloning Kit			
10 ligations and 2 test insert ligations	10 µl predigested pDual vector (20ng/µl), Test insert [CAT (chloramphenicol-resistance gene)], *Eam*1104 I restriction enzyme, 10X Universal buffer, T4 DNA ligase, T4 DNA ligase dilution buffer, 10X ligase buffer, rATP, XL1-Blue supercompetent cells, pUC18 control plasmid, β-mercaptoethanol, BL21(DE3) competent cells	• Includes all reagents to clone and transform your gene of interest • Predigested with *Eam*1104 for Seamless cloning • Includes BL21(DE3) cells	#214500

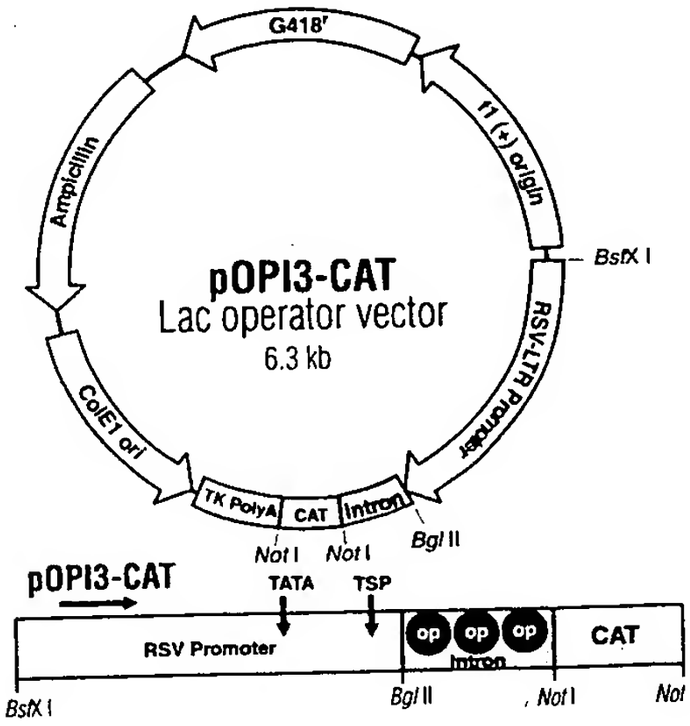
LacSwitch® II Inducible Mammalian Expression System

- Inducible method for controlling gene expression in eukaryotic cells*
- pCMVLacI has CMV promoter** for tighter repression of the inserted gene of interest
- pOPRSVI/MCS has 8 unique sites for directional insertion of gene of interest
- IPTG inducer has no adverse effects in eukaryotic cells
- Rapid induction within 4-8 hours

APPLICATIONS
• Uses *lac* operon to control gene expression in eukaryotic cells • Investigation of gene's function by reversibly turning genes on and off • Ideal for studies of cytotoxic genes
SELECTION
• pCMVLacI: Bacterial selection: Ampicillin; Mammalian selection: Hygromycin • pOPRSVI/MCS: Bacterial selection: Ampicillin; Mammalian selection: G418 • pOPI3-CAT: Bacterial selection: Ampicillin; Mammalian selection: G418
REPLICATION ORIGINS
• F1 filamentous phage: Origin of replication allows recovery of single-stranded DNA • ColE1 origin: Plasmid origin of replication used in *E. coli*
PROMOTERS and EXPRESSION
• pCMVLacI: CMV immediate early promoter drives mammalian expression • pOPRSVI/MCS and pOPI3 CAT: RSV-LTR
CLONING SITES
• pOPRSVI/MCS: *Kpn* I, *Xho* I, *Cla* I, *EcoR* V, *Sma* I, *Spe* I, *Xba* I and *Not* I; pOPI3-CAT: Unique sites for removal of RSV promoter, unique *Not* I site for insertion of gene of interest







Improved System

The LacSwitch® II inducible mammalian expression system*** consists of the pOPI3-CAT, pCMVLacI, and pOPRSVI/MCS vectors, which dramatically improve the system's performance and versatility over the original system. Lac repressor protein, produced from the pCMVLacI vector, blocks transcription by binding the Lac repressor protein to a specific DNA sequence (the operator) in the pOPI3-CAT and pOPRSVI/MCS vectors. IPTG, which has no adverse effect in eukaryotic cells, decreases the binding affinity of the Lac repressor protein to the operator sequences, triggering transcription and expression of the inserted gene. The LacSwitch II system allows induction of the gene of interest within 4-8 hours.

Tight Repression

The pCMVLacI vector provides greater repression of the gene of interest due to the increased strength of the CMV promoter. High expression of the *lacI* gene provides 2- to 10-fold tighter repression of the gene of interest than the original p3'SS vector. A nuclear localization sequence (NLS) directs expression of Lac repressor to the nucleus.

Directional Insertion of the Gene of Interest

The multiple cloning site from pBluescript® II SK was inserted into pOPRSVI to create the pOPRSVI/MCS vector, for directional insertion of the gene of interest and decreased screening time. T3 and T7 promoters on the ends of the MCS in the pOPRSVI/MCS vector allow easy sequencing and verification of positive inserts using T3 and T7 primers.

Removal of the RSV Promoter

The pOPI3-CAT operator vector is included to replace the RSV-LTR promoter with a promoter of interest. This vector contains three operators in the intron and unique restriction sites for removal of the RSV promoter.

REFERENCES

1. DuCoeur, L.C., Wyborski, D.L., and Short, J.M. (1992) *Strategies* 5: 70-72.
2. Wyborski, D.L., and Short, J.M. (1991) *Nucleic Acids Res.* 191: 4647-4653.
3. Fieck, A., Wyborski, D.L., and Short, J.M. (1992) *Nucleic Acids Res.* 20: 1785.
4. Wyborski, D.L., DuCoeur, L.C., and Short, J.M. (1996) *Environ. Mol. Mut.* 28: 447-458.

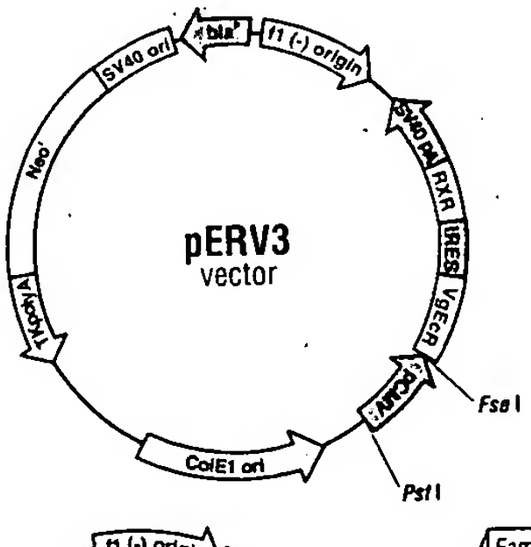
* See license reference 25 on page 324.
** See license reference 4 on page 324.
*** U.S. Patent No. 5,589,392. See license reference 25 on page 324.

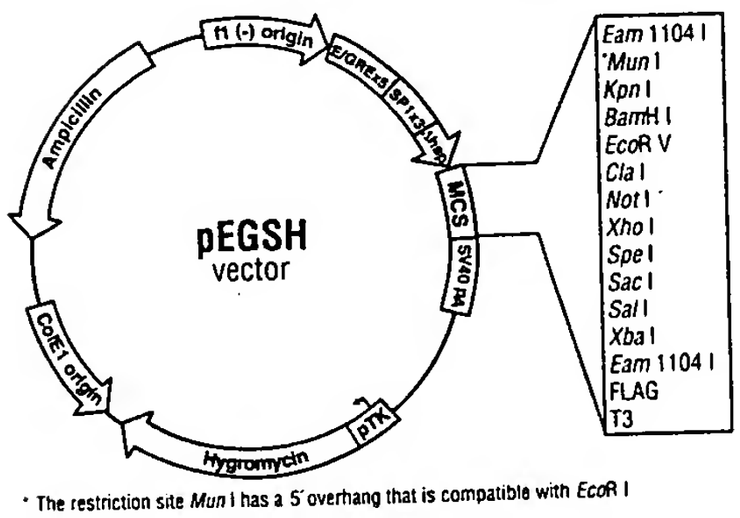
LacSwitch® II Inducible Mammalian Expression System

CONTENTS	DESCRIPTION	CATALOG #
100 µg pCMVLacI vector, 10 µg pOPRSVI/MCS vector, 10 µg pOPI3CAT vector, 1 g IPTG, Host strain: XL1-Blue MR	• Improved system for controlling gene expression in eukaryotic cells • Includes a eukaryotic Lac-repressor-expressing vector and two *lac*-operator vectors	#217450

Complete Control® Inducible Mammalian Expression System

- Dose-responsive inducible mammalian expression
- Induction as high as 1,700 fold
- Tightly repressed
- Novel IRES site allows expression of both receptor subunits from the same CMV promoter
- Retroviral and plasmid gene delivery systems available
- Stable receptor cell lines available





* The restriction site *Mun* I has a 5´ overhang that is compatible with *Eco*R I

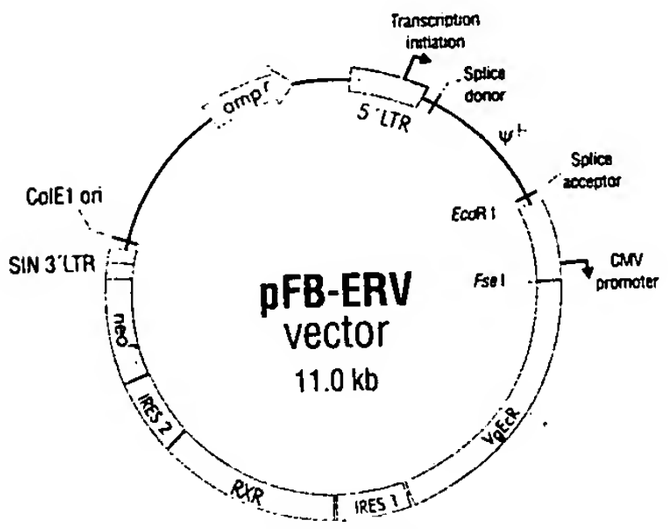
APPLICATIONS
• Control expression of heterologous gene products in mammalian cells • Retroviral system available for gene delivery to difficult to transfect cells
SELECTION
• pERV3 plasmid: Bacterial: Kanamycin; Mammalian: G418 • pEGSH plasmid: Bacterial: Ampicillin; Mammalian: Hygromycin • pFB-ERV retroviral vector: Bacterial: Kanamycin or Ampicillin; Mammalian: G418 • pCFB-EGSH retroviral vector: Bacterial: Ampicillin; Mammalian: Hygromycin
REPLICATION ORIGINS
• pERV3 and pEGSH plasmids: F1 filamentous phage: Origin of replication allows recovery of single-stranded DNA; ColE1 origin: Plasmid origin of replication used in *E. coli*; SV40 origin: Mammalian origin of episomal replication requires expression of SV40 T antigen. • pFB-ERV and pCFB-EGSH retroviral vectors: ColE1 bacterial origin of replication
PROMOTERS and EXPRESSION
• pERV3 plasmid and pFB-ERV retroviral vector: CMV immediate early promoter for mammalian expression • pEGSH plasmid and pCFB-EGSH retroviral vector: SP1/minimal promoter

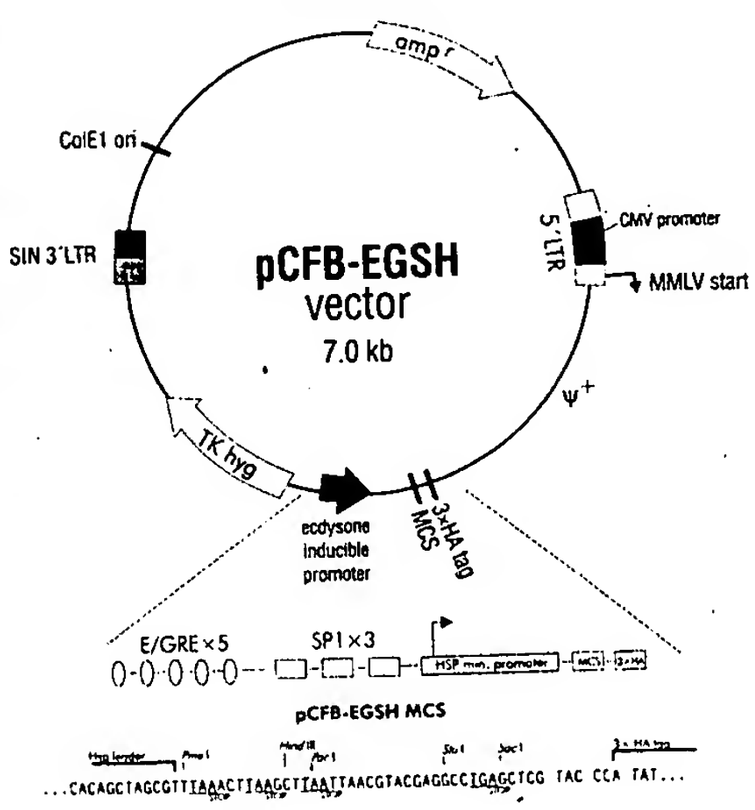
Tight Control

The Complete Control® inducible mammalian expression system* is designed to provide the tightest control of expression available in mammalian cells. The system is based on a synthetic ecdysone-inducible receptor and a synthetic receptor recognition element that modulates expression of your gene of interest. The artificial nature of the receptor and the recognition element ensures that endogenous host transcription factors and genes will not be activated, providing extremely low background in the system. In addition, the receptors are bound to the recognition element in the absence of inducer, further reducing background expression. After addition of ponasterone A, a conformational change of the receptor subunits removes transcription repressors and recruits transcriptional machinery to activate transcription.

Powerful Induction

Induction is fast and potent; in just 20 hours we have observed as high as 1,747-fold induction in transient assays and 1,030-fold induction in a double-stable cell line. Using a range of ponasterone A gives a linear dose response curve, allowing the amount of induction to be easily moderated (data not shown). Two vectors are used to control expression in the Complete Control system. The synthetic receptor is constitutively expressed as a heterodimer consisting of the ecdysone receptor (EcR) and the retinoid-X-receptor (RXR). Both subunits of the receptor are produced from the pERV3 vector, using an internal ribosomal reentry site (IRES)** to allow expression of both proteins from the same CMV promoter.*** This unique design allows expression of the heterodimer receptor in a wide variety of cell lines. The expression vector, pEGSH, is designed for easy detection of expressed protein by either RNase protection assay using T3 antisense probes or by western analysis using the FLAG® **** epitope.





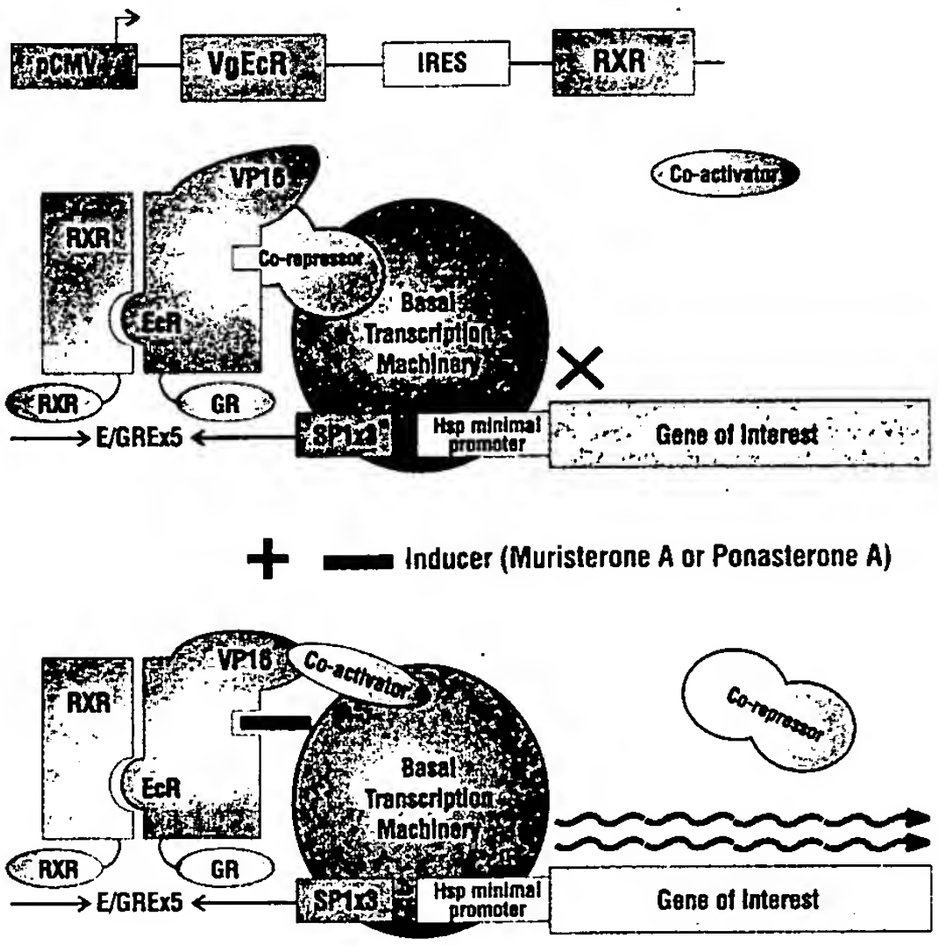
Retroviral System for Gene Delivery to Difficult-to-Transfect Cells

We have developed a retroviral version of the Complete Control inducible mammalian expression system to expand its utility. Gene delivery using retroviruses often yields transduction efficiencies close to 100%, and the proviral copy number can be easily controlled by varying the multiplicity of infection (MOI). This latter feature is particularly important for inducible systems, for which low basal expression and high induction ratios are affected by copy number. Thus, transduction of the target cell with virus at an optimal MOI should yield a high frequency of clones capable of mediating desirable expression profiles without exhaustive colony screening. The two retroviral vectors are pFB-ERV, an MMLV-based replication-defective retroviral vector that delivers the ecdysone receptor proteins RXR and VgEcR, and the pCFB-EGSH retroviral vector containing the inducible cassette. Used together, we have attained induction ratios of >1,000-fold in tissue culture cells (data not shown).

Save Time with Stable Cell Lines

The best method to create double-stable cell lines is sequentially, first establishing pERV3 stable cell lines and selecting the line that gives the highest induction and the lowest backgrounds using the pEGSH-luc control vector in transient assays. Then, use this line to create a double-stable cell line using the pEGSH expression construct containing your gene of interest. Stratagene has eliminated the first step of this process with a collection of pERV3 stable cell lines, derived from CHO, NIH3T3 and 293 cells. Each cell line is tested for viability and functionally tested in a transient assay using the pEGSH-luc control vector and monitoring luciferase expression after induction with ponasterone A.

* See license reference 14 on page 324.
** See license reference 15 on page 324.
*** See license reference 4 on page 324.
**** See license reference 18 on page 324.



The Ecdysone-Inducible Expression System

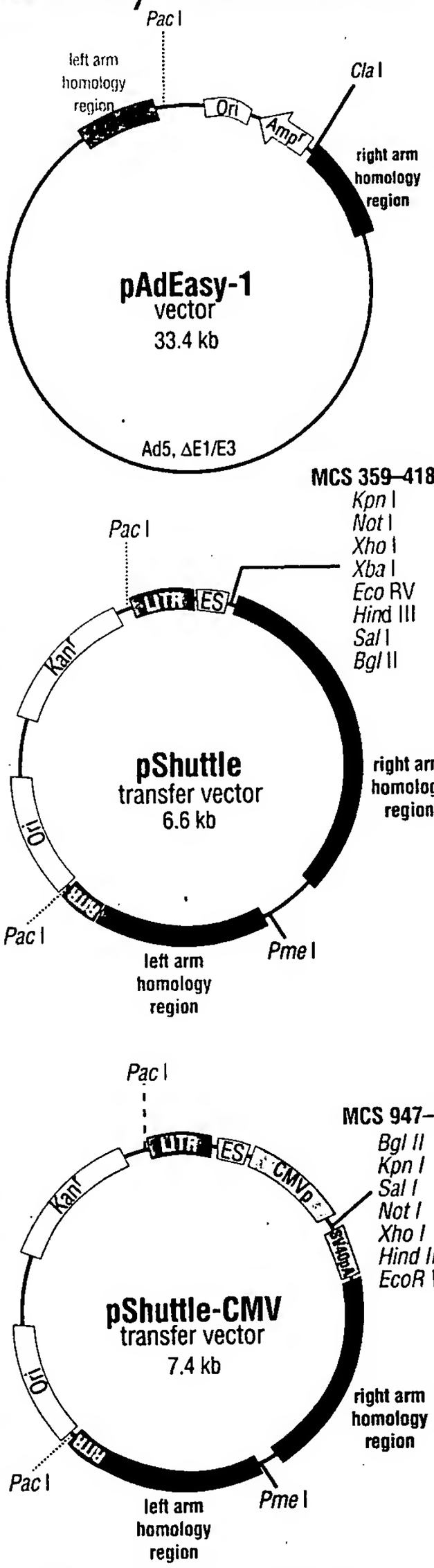
The nuclear receptor proteins RXR and VgEcR are coexpressed from the CMV promoter VgEcR: hybrid nuclear receptor comprised of the ecdysone receptor (EcR) ligand-binding and dimerization domains, the VP16 transcriptional activation domain and the glucocorticoid receptor (GR) DNA binding domain. The heterodimeric ecdysone receptor remains bound to five copies of the E/GRE recognition element located upstream of a minimal promoter in the inducible expression cassette. The inducible promoter remains transcriptionally silent until induction with the ecdysone analogs muristerone A or ponasterone A. Interaction between the inducer and the EcR ligand-binding domain results in the recruitment of coactivator(s) and, thus, transcriptional activation that can reach over three orders of magnitude.

Complete Control® Inducible Mammalian Expression System

	CONTENTS	FEATURES	CATALOG #
Complete Control® Vector Kit	pERV3 vector, pEGSH vector, 1 mg ponasterone A, Sequencing primers		#217468
pERV3 vector	20 µg vector Sequencing primers	• Receptor expression vector • CMV promoter easily replaced to confer cell-type specificity to receptor expression	#217460
pEGSH vector		• Ecdysone-inducible vector • MCS contains restriction sites positioned for directional cloning of inserts derived from Lambda ZAP®-derived cDNA vectors, λgt10, λgt11, HybriZAP® vectors and other two-hybrid libraries • Monitor gene expression using α-FLAG® immunoprecipitation or T3 antisense RNA probes	#217461
Complete Control® Retroviral Inducible Mammalian Expression System			
pFB-ERV retroviral vector	20 µg vector 20 µg pCFB-EGSH-Luc control vector	• Receptor expression vector • CMV promoter easily replaced to confer cell-type specificity to receptor expression	#217564
pCFB-EGSH retroviral vector		• Ecdysone-inducible vector • MCS contains restriction sites positioned for directional cloning of inserts derived from Lambda ZAP®-derived cDNA vectors, λgt10, λgt11, HybriZAP® vectors and other two-hybrid libraries • Monitor gene expression using α-HA immunoprecipitation	#240028
Stable Cell Lines			
ER-CHO cell line	1 x 10^6 cells		#222100
ER-NIH3T3 cell line	1 x 10^6 cells		#222105
ER-293 cell line	1 x 10^6 cells		#222110
Ponasterone A	1 mg		#217467

VIRAL EXPRESSION

AdEasy™ Adenoviral Vector Systems



AdEasy™ System Plasmid Vectors
pShuttle is a transfer vector with a multiple cloning site into which an entire expression cassette can be inserted. The transfer vector pShuttle-CMV contains a multiple cloning site sandwiched between the CMV promoter and the SV40 polyadenylation signal. The pAdEasy-1 plasmid contains most of the human adenovirus serotype 5 genome with deletions in the E1 and E3 regions. Upon homologous recombination between a shuttle vector and pAdEasy-1, a recombinant adenoviral plasmid is generated in which the expression cassette is inserted into the original E1 region of the adenovirus genome.

- Easiest way to efficiently gene transfer and express a wide variety of genes in mammalian cells
- AdEasy™ XL kit includes all components of original AdEasy system, plus two new components for even more convenient and easier adenovirus production
- Rapid generation of adenoviral vectors by homologous recombination in *E.coli*
- Save weeks of work and avoid plaque purification
- Infects dividing and non-dividing cells
- Up to 100% transduction efficiency, even on difficult-to-transfect cell lines
- Larger insert capacity (7.5 kb)
- Lower toxicity than non-viral methods of gene delivery

APPLICATIONS
• High-level protein expression in mammalian cells • Gene function studies • Gene delivery to difficult-to-transfect cells
SELECTION
• pShuttle, pShuttle-CMV, pShuttle-CMV-LacZ: Bacterial selection: Kanamycin; Mammalian selection: G418 • pAdEasy-1: Bacterial selection: Ampicillin
REPLICATION ORIGINS
• All plasmids: pBR322
PROMOTERS and EXPRESSION
• pShuttle-CMV, pShuttle-CMV-LacZ: CMV immediate early promoter* for mammalian expression and SV40 polyadenylation signal provides signals required for termination of mammalian transcription and translation. • pShuttle: No promoter, allows insertion of desired expression cassette

Homologous Recombination Saves Time

AdEasy™ adenoviral vector systems** simplify the production of recombinant adenoviruses for versatile gene delivery and expression. The construction of a recombinant adenoviral vector using the AdEasy system is a two-step process in which the desired expression cassette is first subcloned into a shuttle vector, and then transferred into the adenoviral genome by homologous recombination in *E.coli*. This method saves you weeks of time and easily selects the recombinant adenoviral vectors compared to traditional methods. The AdEasy system was developed by T.C. He et al. as an easy alternative to traditional methods of generating recombinant adenoviral vectors.

High-Level Transient Protein Expression

Adenoviruses are capable of infecting a broad range of cell types and infection is not dependent on active host cell division. High titers and high-level gene expression can be generated, important considerations for protein production techniques in mammalian cells. Adenoviral vectors can be used to over-express recombinant proteins in human cells instead of the traditional *E.coli* expression system. This avoids the *E.coli* system's problems with expressing different types of heterologous proteins, including lack of posttranslational modifications, incorrect folding, proteolytic degradation and inefficient secretion. Since the AdEasy system employs human adenovirus and human host cell lines, the proteins expressed are abundant and have the correct posttranslational modification and folding. Use adenovirus gene delivery when transient, high-level protein expression is desired.

Faster, Easier Adenovirus Gene Delivery

In the AdEasy XL system, we have pretransformed pAdEasy-vector into BJ5183 cells to create BJ5183-AD-1 cells. As a result, only pShuttle vector, into which the gene of interest is cloned, is transformed. Having the adenoviral backbone pre-transformed into *E. coli* significantly increases the probability of generating a recombinant adenoviral vector with the desired expression cassette.[3]

In the original AdEasy system, the cDNA or expression cassette of interest is cloned into either of two shuttle vectors, pShuttle or pShuttle-CMV. Once constructed, the shuttle vector is linearized with *Pme I* and co-transformed into BJ5183 cells together with pAdEasy-1 vector, the supercoiled viral DNA plasmid.

In both AdEasy systems, transformants are selected on kanamycin and recombinants are subsequently identified by restriction digest. Once a recombinant is identified, it is produced in bulk using the recombination-deficient XL10-Gold® strain. Purified recombinant adenoviral plasmid DNA is then linearized by *Pac I* to expose its inverted terminal repeats (ITR) and transfected into HEK293 cells where deleted viral genes necessary for virus assembly are complemented in vivo.

AdEasy™ XL Adenoviral Vector System

NEW

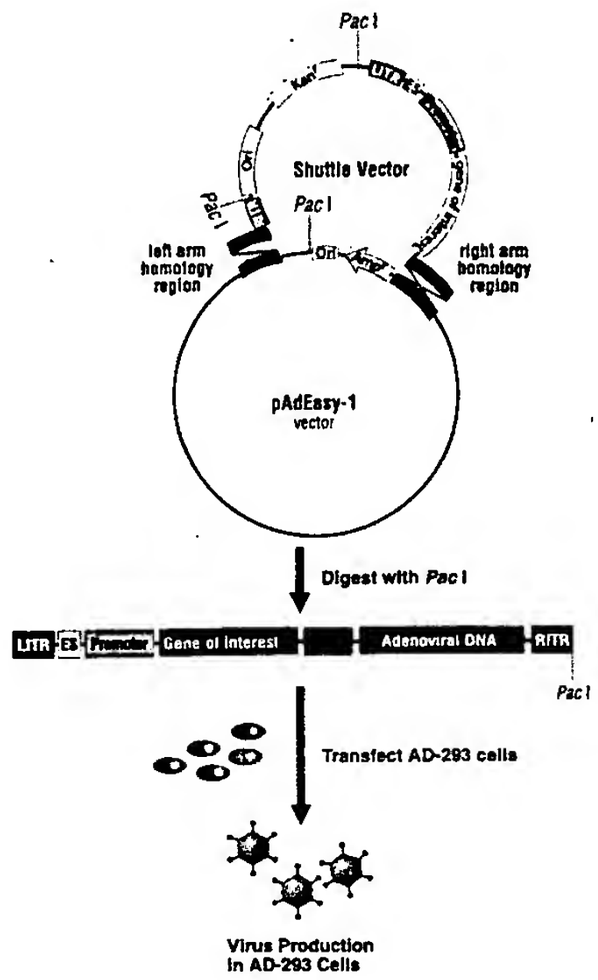
- New kit for even more convenient and easier adenovirus production
- Specialty competent cells result in 3-fold more colonies containing positive recombinants
- Adherent AD-293 cells for convenient virus production
- Both additional components are also available separately

Two new additions in the AdEasy™ XL adenoviral vector system make adenovirus production easier than ever! The new competent cells, BJ5183-AD1, are pretransformed with the pAdEasy-vector providing a 3-fold improvement in your chance of selecting a clone with the desired recombinant. Adherent AD-293 cells are included for convenient virus production. Both components are available in the new kit and separately.

AdEasy™ Adenoviral Vector System

- Original system for fast and easy production recombinant adenovirus

The original AdEasy system requires transformation of two plasmids: pAdEasy-1 and pShuttle into electroporation-competent cells.



How the AdEasy™ XL System Works

The gene of interest or expression cassette is first cloned into a shuttle vector (either pShuttle or pShuttle-CMV). The resultant plasmid is linearized by *Pme* I and cotransformed into *E. coli* strain BJ5183-AD-1 which contains the pAdEasy vector. Recombinant adenoviral plasmids are selected on kanamycin and confirmed by restriction digest. The recombinant adenoviral plasmid is then digested with *Pac* I and transfected into AD-293 cells where they are packaged into virus particles.

REFERENCES

1. He, T.C., et al. (1998) *Proc. Natl. Acad. Sci.* 95: 2509-2514.
2. Chartier C., Degryse E., et al. (1996) *J. Virol.* 70(7): 4805-10.
3. Zeng, M., et al. (2001) *BioTechniques* 31: 261-262.

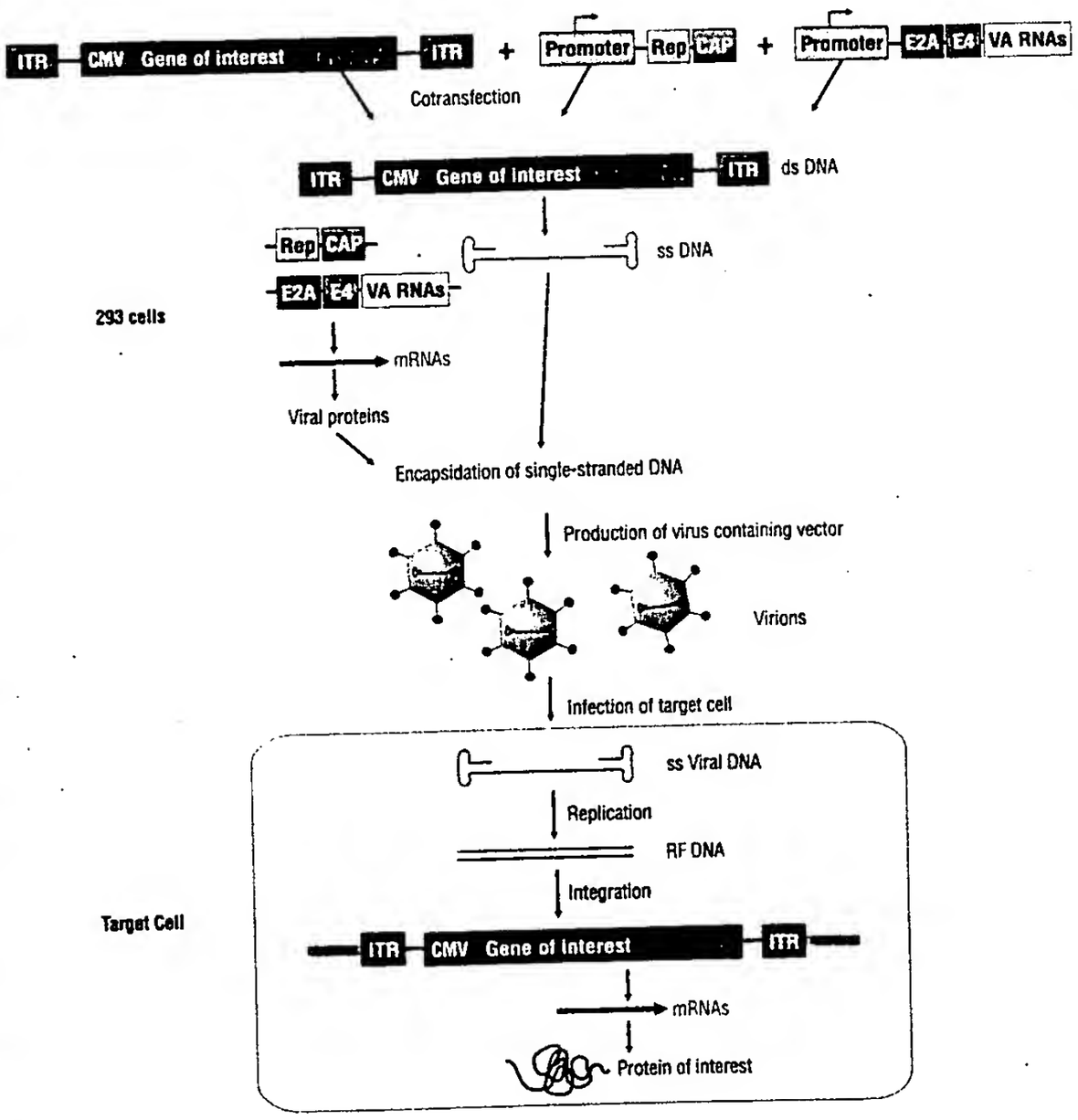
* See license reference 4 on page 324.
** See license references 3 and 5 on page 324. The AdEasy system was developed by T.C. He et al.

AdEasy™ Adenoviral Vector Systems

CONTENTS	DESCRIPTION		CATALOG#
AdEasy™ XL Adenoviral Vector System			
AdEasy XL System	pShuttle vector, pShuttle-CMV vector, pShuttle-CMV-lacZ control vector, BJ-5183-Ad1 electroporation-competent cells, Transformation control plasmid, XL10-Gold® ultracompetent cells, XL10-Gold® ß-mercaptoethanol, pUC18 DNA control plasmid, Ad-293 cells		#240010
AD-293 cells	1×10^6	• For adenovirus vector packaging	#240085
BJ5183-AD1 electroporation-competent cells	5 x 100 µl	• Pretransformed with pAdEasy-1adenoviral vector backbone for 3-fold improvement of recombinant adenovirus production	#200157
AdEasy™ Adenoviral Vector System			
AdEasy System	pAdEasy-1 vector, pShuttle vector, pShuttle-CMV vector, pShuttle-CMV-LacZ vector, BJ5183 electroporation-competent cells, XL10-Gold ultracompetent cells, XL10-Gold ß-mercaptoethanol mix, pUC18 DNA control plasmid		#240009
BJ5183 electroporation-competent cells	5 x 100 µl	• Competent cells for homologous recombination	#200154
AdEasy™ Adenoviral Vectors			
pAdEasy-1 vector	2.5 µg, 100 ng/µl in TE buffer	• Replication-deficient adenoviral vector	#240005
pShuttle vector	20 µg, 1 µg/µl in TE buffer	• Cloning vector for expression cassette of choice	#240006
pShuttle-CMV vector	20 µg, 1 µg/µl in TE buffer	• Cloning vector for use with CMV promoter	#240007
pShuttle-CMV-LacZ control vector	10 µg, 1 µg/µl in TE buffer	• Control vector with LacZ reporter	#240008
MBS Mammalian Transfection Kit	2.5 M $CaCl_2$, 2x BBS (pH 6.95), Modified bovine serum (MBS), pCMV ß-gal control	• For highest viral titer use this kit to transfect HEK293 cells	#200388

AAV Helper-Free System

- Safe, helper-free system—no adenovirus required
- Stable and efficient integration of DNA into host genome
- Broad host range
- Infects both dividing and non-dividing cells
- High titers, up to 10^{11} - 10^{12} viral particles (vp)/ml with concentration
- Up to 100% transduction efficiency, even on difficult-to-transfect cell lines
- Lower toxicity vs. non-viral methods of gene delivery
- Proteins expressed with the correct post-translational modifications and folding



How the AAV Helper-Free System Works

Viral-Based System for Gene Delivery

The AAV Helper-Free System* is a viral-based gene delivery system that delivers high-efficiency gene delivery to a broad range of hosts.[1,2] The AAV system simplifies the study of gene expression in a native host by overcoming the problem that many of these hosts are difficult or impossible to transfect. Using traditional transfection methods, these studies are limited to hosts which easily accept DNA.

Efficient Gene Delivery, Stable Expression

Recombinant adeno-associated virus (AAV) is a proven research and therapeutic tool[3]. This system is used to introduce genes into cells for gene expression or gene therapy studies. Using the AAV system, genes can be delivered into a wide range of hosts including many different human and non-human cell lines or tissues. Since gene delivery is possible in mammalian systems, expressed proteins have all of the necessary posttranslational modifications and folding. Recombinant AAV are capable of transducing a broad range of cell types and transduction is not dependent on active host cell division. High titers, $>10^8$ vp/ml, are easily obtained in the supernatant and $\geq 10^{11}$ to 10^{12} vp/ml with further concentration. The gene of interest is integrated into the host genome so expression is long term and stable.

Helper-Free, Safe System

AAV is naturally replication-deficient and normally requires coinfection with an unrelated helper virus, like adenovirus, to generate AAV virions. This novel system uses a vector containing the necessary genes from adenovirus (pHelper vector) to induce the lytic phase of AAV producing recombinant, replication-defective AAV virions ready to deliver a gene of interest to target cells. Eliminating the need for helper virus coupled with the fact that the AAV virus has never been associated with any known human diseases gives this system a high biosafety profile.

Generating Recombinant, Replication-Deficient AAV

The system is comprised of four vectors; a cloning vector, a replication-deficient AAV vector and two vectors that carry necessary genes for generating infectious viral particles. The pCMV-MCS cloning vector has a versatile multiple cloning site for insertion of the gene of interest. Once the gene of interest is cloned into this vector, the expression cassette is removed using the flanking *Not* I sites. The expression cassette is inserted into the pAAV-LacZ vector that is also cut at the *Not* I sites and the LacZ cassette is removed. The double cloning step allows for applications like site-directed mutagenesis to be performed in the more stable pCMV-MCS vector whereas these applications could cause recombination if performed directly in the pAAV-LacZ vector due to the ITRs. The recombinant pAAV-LacZ vector is cotransfected into a packaging cell line with the pAAV-RC and pHelper vectors. Replication-deficient, recombinant virions are produced. The cell lysate is used to transduce target cells. The gene of interest is integrated into the host genome for stable expression.

pCMV-MCS Vector

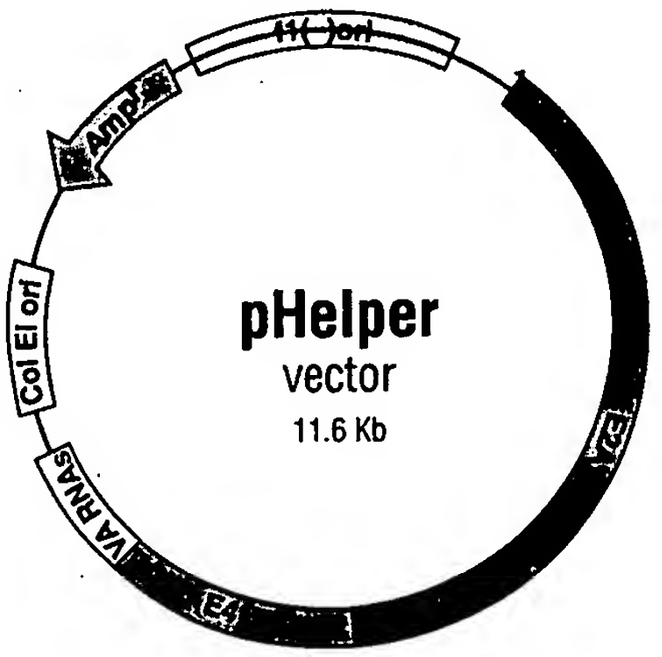
The pCMV-MCS is a cloning vector. The gene of interest is cloned into the multiple cloning site. After cloning, the expression cassette is removed from this vector using the flanking *Not* I sites for subsequent cloning into the pAAV-LacZ vector.

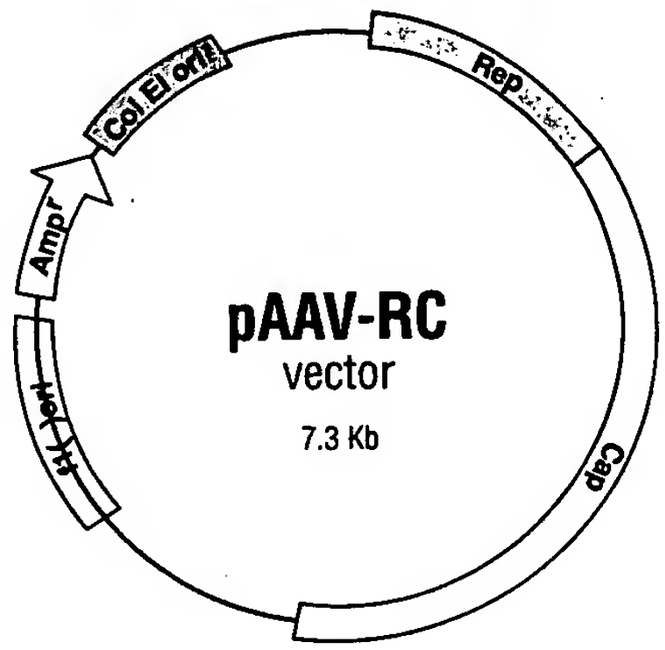
pAAV-LacZ Vector
The pAAV-LacZ is the replication-deficient AAV vector. This vector serves two purposes; first, as a cloning vector; second, as a control vector. As a cloning vector, this vector is cut with *Not* I, the LacZ cassette is lost, and the expression cassette harboring the gene of interest from the pCMV-MCS vector is cloned into the complementing *Not* I sites. The recombinant vector is cotransfected into a viral packaging cell line for production of recombinant, replication-deficient AAV virions. This vector is also a control using the LacZ reporter enzyme.

pAAV-RC Vector
This vector harbors the *cap* and *rep* genes which encode for the capsid and DNA replication proteins required to make infectious virions. This vector is cotransfected into the packaging cell line for production of recombinant, replication-deficient AAV virions.

pHelper Vector
This vector carries the adenovirus genes (E2A, E4 and VA RNAs) required for inducing the lytic phase of AAV. This vector is cotransfected into the packaging cell line to produce recombinant, replication-deficient AAV virions.

pAAV-hrGFP Vector
This is an alternate positive control vector containing the humanized *Renilla* GFP (hrGFP) reporter gene. Use it for easy detection of expression in vivo using fluorescence microscopy, fluorescence-activated cell sorting (FACS) analysis or fluorometry.





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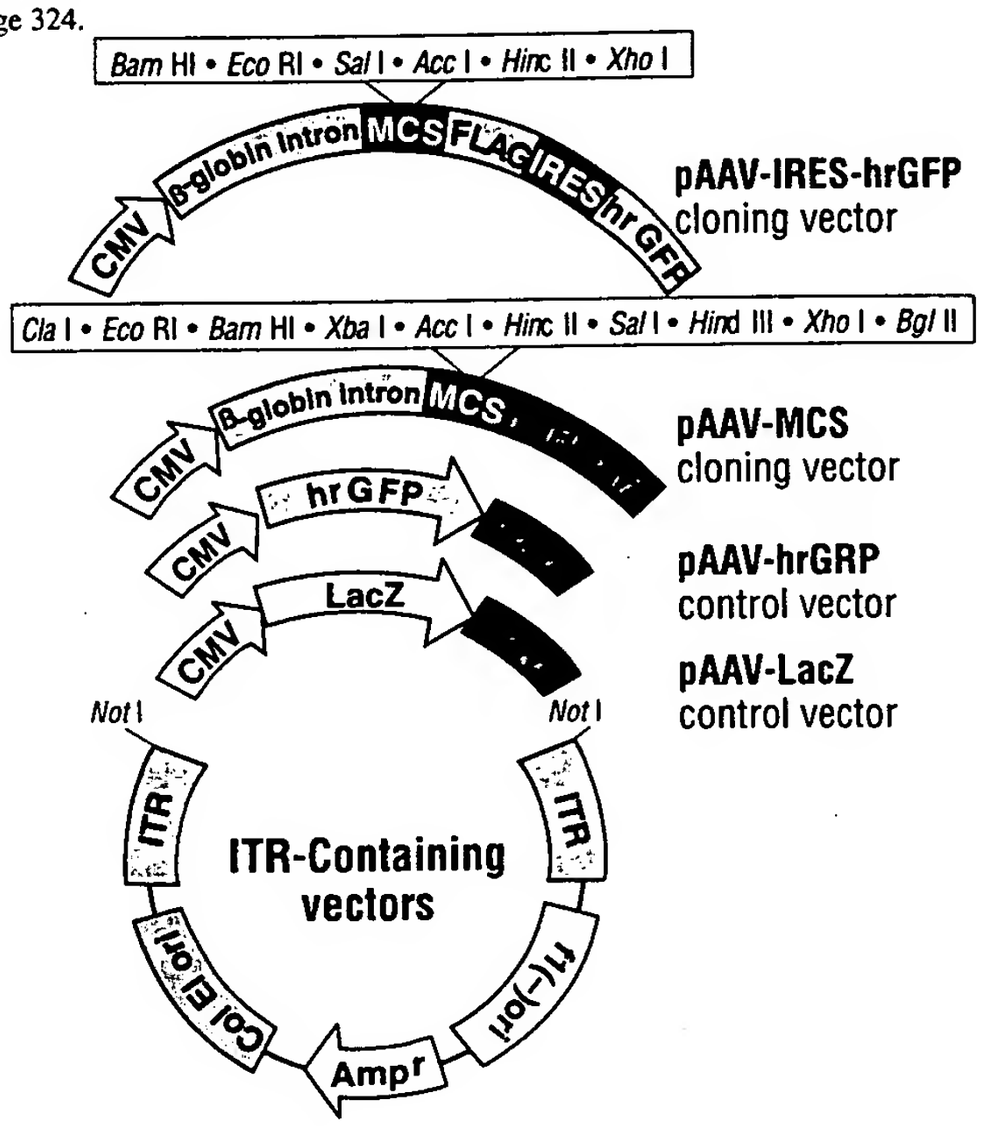
1. Matsushita, T., et al. (1998) *Gene Therapy* 5: 938-945
2. Xiao, X., Li, J. and Samulski, R.J. (1998) *J. of Virol.* 72 (3): 2224-2232
3. Kay, M. A., et al. (2000) *Nature Genetics* 24: 257-261

* See license reference 1 on page 324.

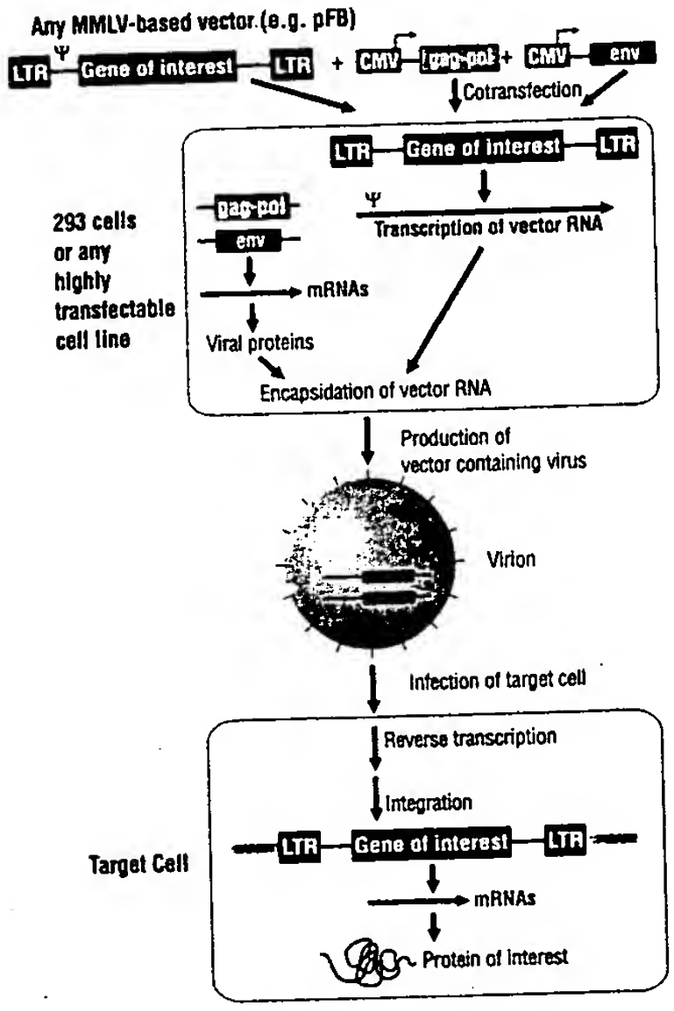


AAV Helper-Free System

	CONTENTS	FEATURES	CATALOG #
Complete AAV System			
pMCS vector	10 µg	• Cloning vector and source of expression cassette	#240071
pAAV-LacZ vector	10 µg	• Expression cassette with gene of interest cloned into replication-deficient AAV vector • Original vector with LacZ is a control vector	
pAAV-RC vector	20 µg	• Carries the *rep* and *cap* AAV genes required to generate recombinant, replication-deficient AAV virions	
pHelper vector	20 µg	• Carries the adenovirus genes required to generate recombinant, replication-deficient AAV virions	
pAAV-hrGFP Vector	20 µg	• Positive control vector containing the humanized *Renilla* GFP (hrGFP) reporter gene	#240074
pAAV-IRES-hrGFP Vector	20 µg	• Clone gene of interest directly into this ITR-containing vector • Express both the recombinant protein and Vitality hrGFP from the same mRNA	#240075

ViraPort® Retroviral Gene Expression System

- Highly efficient gene delivery—up to 100% transduction efficiency
- Controls copy number and allows easy identification of cDNA targets—simply adjust the multiplicity of infection (MOI) to meet your needs
- Easy expression assay
- System includes retroviral vectors, VPack plasmid vectors, reporter vectors, transduction-ready VSV-G retroviral supernatants and premade retroviral libraries
- More information on page 106



How the ViraPort® System Works
Co-transfect three vectors: these include a *gag-pol*-expressing vector; the retroviral expression vector; and one of a choice of 4 envelope (*env*)-expressing vectors. The choice of *env*-expressing vector is based on the range of cell types you want to use to screen your library. Upon infection of the target cell, the viral RNA molecule is reverse transcribed and the cDNA of interest, flanked by the LTRs, is integrated into the host DNA. Because the vector itself carries none of the viral proteins, once a target cell is infected, the LTR expression cassette is incapable of proceeding through another round of virus production. Screening is done based on protein function.

Triple Transfection Ensures Production of Replication-Incompetent Virus

In the ViraPort® retroviral gene expression system,* all *cis* and *trans* elements required to produce infectious virus are separated onto 3 plasmids, with minimal or no sequence overlap. This triple transfection makes the ViraPort retroviral gene expression system much safer than other systems. In most stable producer cell lines or vector-based systems there is a large degree of homology between the packaging vector and the retroviral expression vector, and a relatively high probability of producing replication-competent retrovirus (RCR) due to homologous recombination between the vectors. With the ViraPort retroviral gene expression system, you don't need to maintain a packaging cell line. To make a virus, all three plasmids are simultaneously transfected using the MBS Mammalian Transfection Kit, and the resulting virions are replication incompetent.

ViraPort® Retroviral cDNA Expression Libraries

- Supplied as complete kits with expression controls and PCR primers
- >1x10⁶ primary clones amplified
- All libraries amplified only once
- Achieve up to 100% transduction efficiency
- Easily control copy number by adjusting the MOI
- See page 152 or **www.stratagene.com** for more information

ViraPort® Reporter Vectors

- Measure transduction efficiency
- Readily assay for gene expression
- Choose from three reporter vectors : hrGFP, for fluorescence assay, lacZ for ß-gal activity or visual staining assays, and luciferase for measuring luciferase activity
- Ideal for use with the ViraPort™ retroviral gene expression system
- Available in two convenient formats: retroviral vector or transduction-ready VSV-G pseudotyped retroviral supernatants

VPack Vectors**

- Produce high-titer, replication-incompetent virus
- For use in a wide range of host cells—choose the *env*-expressing vector based on cell line
- Use these packaging vectors to achieve viral titers that are consistently > 10^7
- Eliminate the need to maintain a packaging cell line

Host Range of VPack env Expression Vectors

	Mouse	Rat	Hamster	Rabbit	Mink	Cow	Cat	Dog	Monkey	Human	Chicken
Ecotropic	+	+	–	–	–	–	–	–	–	–	–
Amphotropic	+	+	+/–	+	+	+/–	+	+	+	+	+/–
10A1	+	+	+	N.D.	+	N.D.	+	+	+	+	N.D.
VSV-G	+	+	+	+	+	+	+	+	+	+	+

* See license reference 5 on page 324.
** See license references 36 and 38 on page 324.

ViraPort® Retroviral Gene Expression System

ViraPort® Retroviral Gene Expression System		See page 107 for ordering information	
VPack Vectors			
ViraPort® Reporter Vectors			
MBS Mammalian Transfection Kit			
2.5 M $CaCl_2$, 2x BBS, pH 6.95, Modified bovine serum (MBS), pCMV β-gal control plasmid	100 transfections in 60-mm dishes	• Ideal for use with the ViraPort retroviral gene expression system • Transiently transfects cells at a frequency of >1 in 10 cells • Generates stable transfectants	#200388
Luciferase Assay Kit	100 reactions	• Quick and sensitive detection of luciferase activity	#219020

YEAST EXPRESSION

pESC Vectors

- Allow expression of 2 different genes in *S. cerevisiae*
- Provide two different epitope tags
- Available with 4 different yeast selective markers

APPLICATIONS
• Protein expression in *S. cerevisiae* • Epitope tagging of expressed proteins • Pull-down experiments
SELECTION
• Bacterial selection: Ampicillin *S. cerevisiae* selection: HIS3, LEU2, TRP1 or URA3
REPLICATION ORIGINS
• ColE1 origin: Plasmid origin of replication used in *E. coli*, 2µ origin
PROMOTERS and EXPRESSION
• GAL1 and GAL10

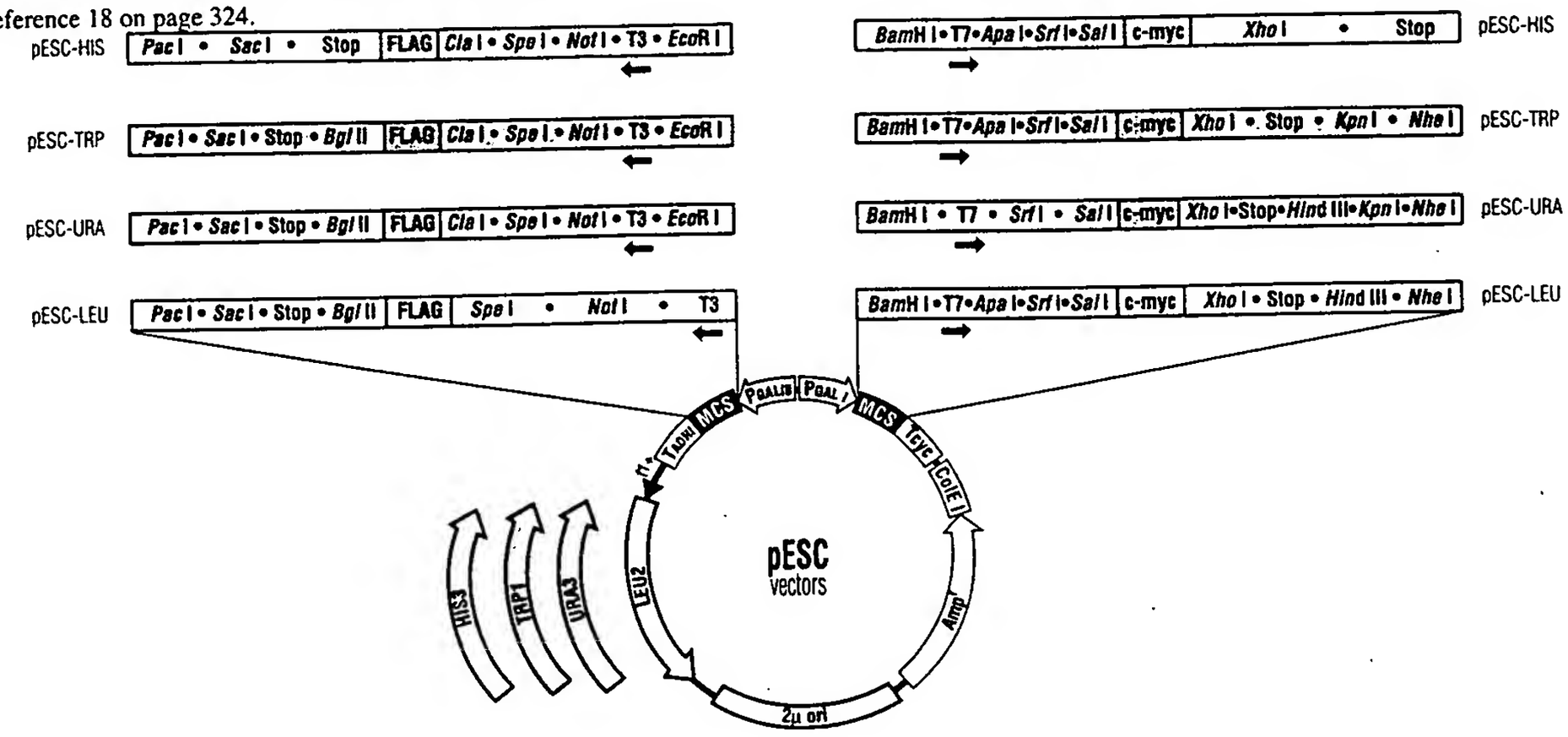
Versatile Yeast Expression

pESC vectors are a series of epitope-tagging vectors for expression and functional analysis of eukaryotic genes in the yeast *S. cerevisiae*. Each vector contains GAL1 and GAL10 yeast promoters in opposing orientation. With these vectors one or two cloned genes can be introduced into a yeast host strain under the control of a repressible promoter. When two genes are co-expressed, protein-protein interactions can be confirmed by immunoprecipitation analysis. These vectors feature an extensive polylinker sequence and the ability to generate end-specific RNA transcripts from T3 and T7 promoters. Each of the pESC vectors contains one of four different yeast-selectable markers (HIS3, TRP1, LEU2, or URA3) in the same vector backbone.

Epitope Tagging

The pESC vectors contain DNA sequences coding for epitope peptides that can be specifically recognized by monoclonal antibodies. A sequence for the FLAG® epitope* (DYKDDDDK) is located in the multiple cloning site (MCS) downstream of the GAL10 promoter; a sequence for the c-myc epitope (EQKLISEEDL) is located in the MCS downstream of the GAL1 promoter. The gene of interest can be inserted in front of the epitope sequence to generate C-terminal tagging or after the epitope sequence for N-terminal tagging. These tags allow the protein of interest to be studied without generating a specific antibody to that protein. The epitope-tagged fusion proteins can be studied in transformed cells using well-characterized antibodies.

* See license reference 18 on page 324.

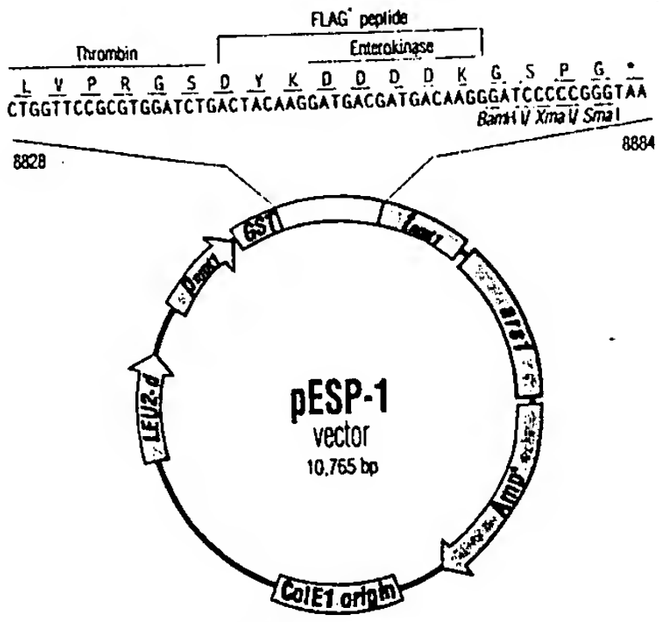


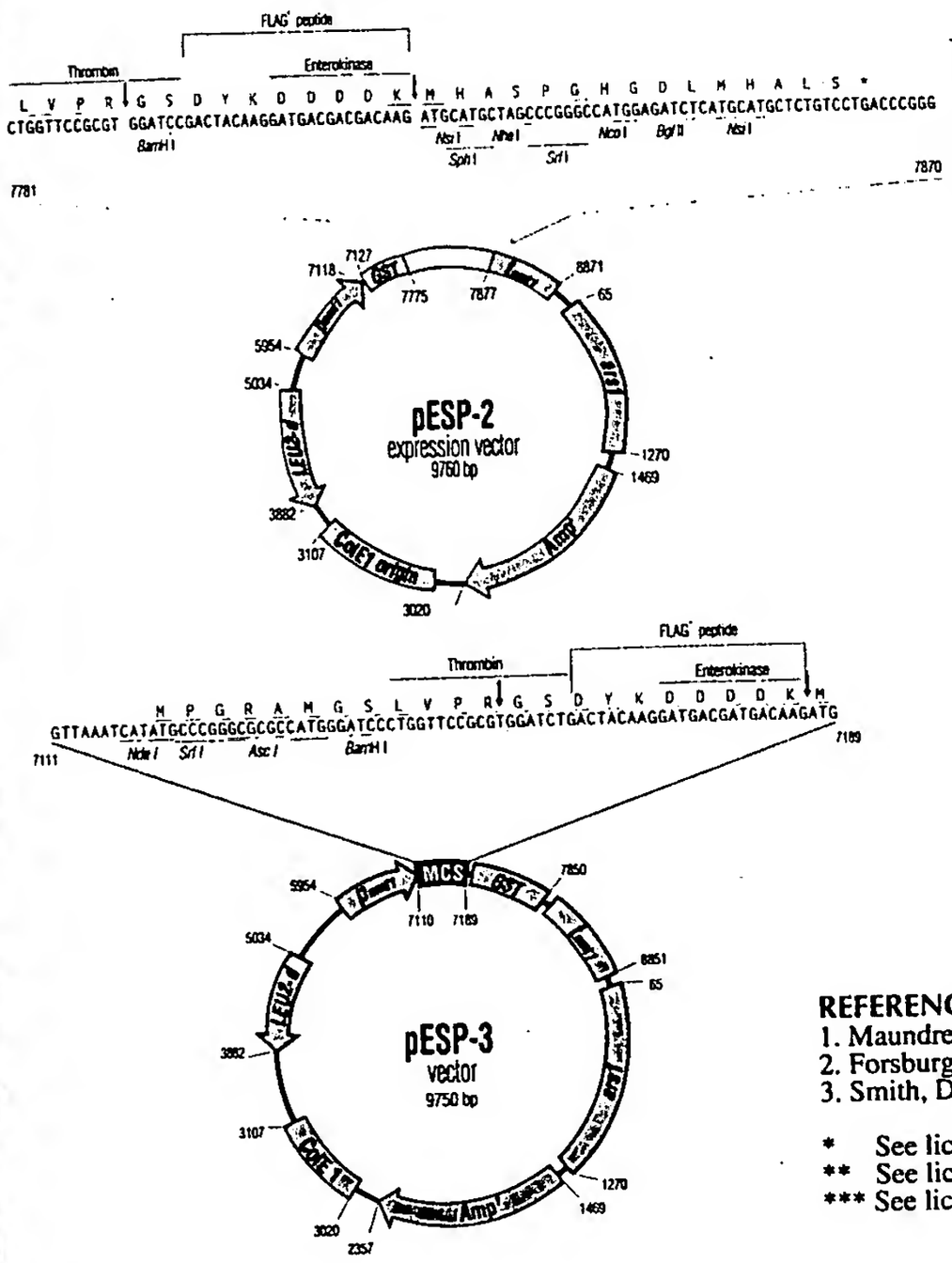
pESC Vectors

	CONTENTS	DESCRIPTION	CATALOG #
pESC-HIS Vector	20 µg supercoiled vector Sequencing primers Yeast host strains	• HIS3 yeast-selectable marker	#217451
pESC-LEU Vector		• LEU2 yeast-selectable marker	#217452
pESC-TRP Vector		• TRP1 yeast-selectable marker	#217453
pESC-URA Vector		• URA3 yeast-selectable marker	#217454
pESC Vectors (set of all four)	20 µg each pESC supercoiled vector Sequencing primers, Yeast host strains	• Includes all four pESC vectors	#217455

ESP® Yeast Protein Expression and Purification System

- Eukaryotic protein expression in *yeast Schizosaccaromyces pombe*
- Capable of many posttranslational modifications
- Single-column purification using GST tag*
- Tight regulation of protein expression
- Provides most reagents for high-level protein expression and purification





Advanced Host with Unique Capabilities

The ESP® yeast protein expression and purification system uses the yeast *Schizosaccharomyces pombe* as the expression host and the glutathione *S*-transferase (GST) peptide as the protein purification tag, for an easy alternative to protein production in *E. coli*. Proteins expressed in *E. coli* may lack proper biological function and antigenicity due to the absence of eukaryotic posttranslational modifications. Eukaryotic proteins expressed in *S. pombe* are more likely to be folded properly, improving the specific activity and minimizing protein-insolubility problems found in *E. coli* expression systems.

Rapid One-Column Purification

The GST affinity tag allows for rapid, near homogeneous, single-column purification of proteins expressed from the pESP vectors. N- or C-terminal tags are available, depending on the pESP vector. Purification of the GST fusion proteins to 90% homogeneity is easily achieved using this procedure. After purification, the GST affinity tag may be removed by proteolytic cleavage with enterokinase** or thrombin. Thrombin cleavage leaves the FLAG® epitope tag*** in the recombinant protein, allowing further analysis when using the pESP-1 or pESP-2 vectors.

Tightly Controlled Expression

Protein expression from the pESP vectors is controlled by the *nmt*1 (no message in thiamine) promoter. The p_{nmt1} is strictly regulated by the concentration of thiamine in the media. The promoter's induction ratio is approximately 300-fold. The *nmt*1 promoter is >80% stronger than the commonly used yeast *adh* promoter. Media for induced and non-induced growth of the SP-Q01 *S. pombe* host is included with the ESP systems.

REFERENCES

1. Maundrell, K. (1990) *J. Biol. Chem* 165: 10857-10864.
2. Forsburg, S. (1993) *Nucleic Acids Res.* 21: 2955-2956.
3. Smith, D.B. and Johnson, K.S. (1988) *Gene* 67: 31-40.

* See license reference 17 on page 324.
** See license reference 7 on page 324.
*** See license reference 18 on page 324.

APPLICATIONS
• High-fidelity PCR cloning
SELECTION
• Bacterial selection: Ampicillin; Yeast selection: LEU
REPLICATION ORIGINS
• ColE1 origin in bacteria; *ars* fragment in yeast
PROMOTERS and EXPRESSION
• *nmt1* promoter of *S. pombe*

ESP® Yeast Protein Expression and Purification System

ESP® Protein Expression and Purification Kit			pESP-1 vector, pESP-1 control vector, Forward and reverse PCR/sequencing primers, XL1-Blue supercompetent cells, *S. pombe* strain, YES and EMM Medium, Acid-Washed Glass Beads, GST Affinity Resin	#251600
ESP® System Components				
pESP-1 Vector			Vector, Forward and reverse PCR/sequencing primers, *S. pombe* strain	#217420
pESP-2 Vector				#217444
pESP-3 Vector				#217445
ESP Protein Purification Components			*S. pombe* strain, YES and EMM Medium, Acid-Washed Glass Beads, GST Affinity Resin	#251605
FLAG® Western Detection Kit			Anti-FLAG M2 antibody, AP conjugated goat anti-mouse antibody, Detection reagents	#200470
Anti-FLAG® M2 Antibody*	(1 mg)	#200472	GST Affinity Resin (10 ml)	#251612
	(200 µg)	#200471	(100 ml)	#251614
Acid-Washed Glass Beads	(100 g)	#200069	Enterokinase (50 U enterokinase, STI agarose)	#251700
YES Medium (growth media)	10 x 35 g	#200066	EMM Medium (for protein expression) 10 x 32 g	#200064
	1 x 1 kg	#200067	1 x 1 kg	#200063
SP-Q01 *S. pombe* Glycerol Stock		#200319		

* See license reference 8 on page 324.

PROKARYOTIC EXPRESSION

- Provide high-level protein expression
- Control expression levels with three derivatives
- Lack OmpT and Lon proteases
- Use with T7- or non-T7 RNA polymerase-based systems*

BL21-CodonPlus® Competent Cells Solve Codon Bias Problem

- Optimized for high-level expression of heterologous proteins
- Express recombinant genes encoding rare codons without additional procedures
- Contain extra copies of genes encoding tRNAs for codons rarely used in *E. coli*

BL21-CodonPlus® strains** help solve the problems related to codon bias, with extra copies of rare *E. coli* tRNA genes to allow high-level expression of many proteins that are difficult or impossible to express in conventional *E. coli* hosts. BL21-CodonPlus®-RIL cells carry extra copies of the argU, ileY, and leuW tRNA genes to resolve the issue of codon bias for organisms with high ATcontent. For reliable protein expression from genomres with high GC content, BL21-CodonPlus®-RP cells have extra copies of argU and proL genes.

BL21-Gold Competent Cells Save 2 Days

- Contain Hte phenotype for increased transformation efficiency
- Produce high-quality miniprep DNA
- Allow direct cloning of many expression constructs

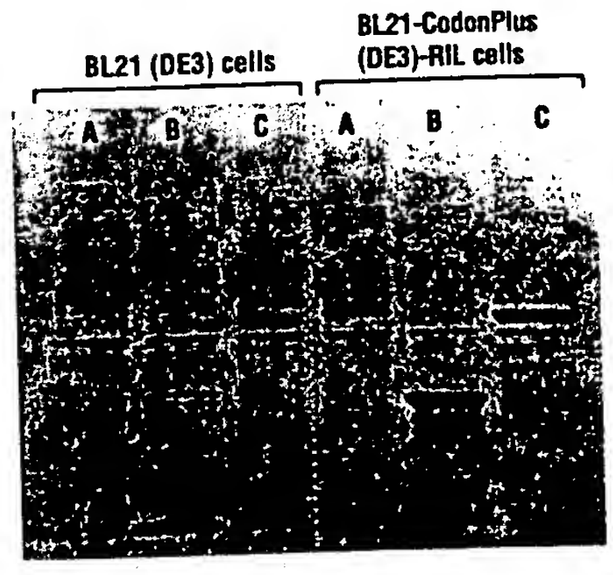
BL21-Gold cells feature the Hte phenotype,*** which contributes to a 100-fold increase in transformation efficiency over the original BL21 cells. The gene-encoding endonuclease I (*endA*) is inactivated to allow direct cloning for most protein expression constructs, saving 2 days of subcloning procedures.

BL21 Cells Are an Economical Alternative

- Used for established expression constructs
- Provide a lower-efficiency, economical alternative

* See license reference 6 on page 324.
** Patent pending
*** Patent pending

BL21 Competent Cells

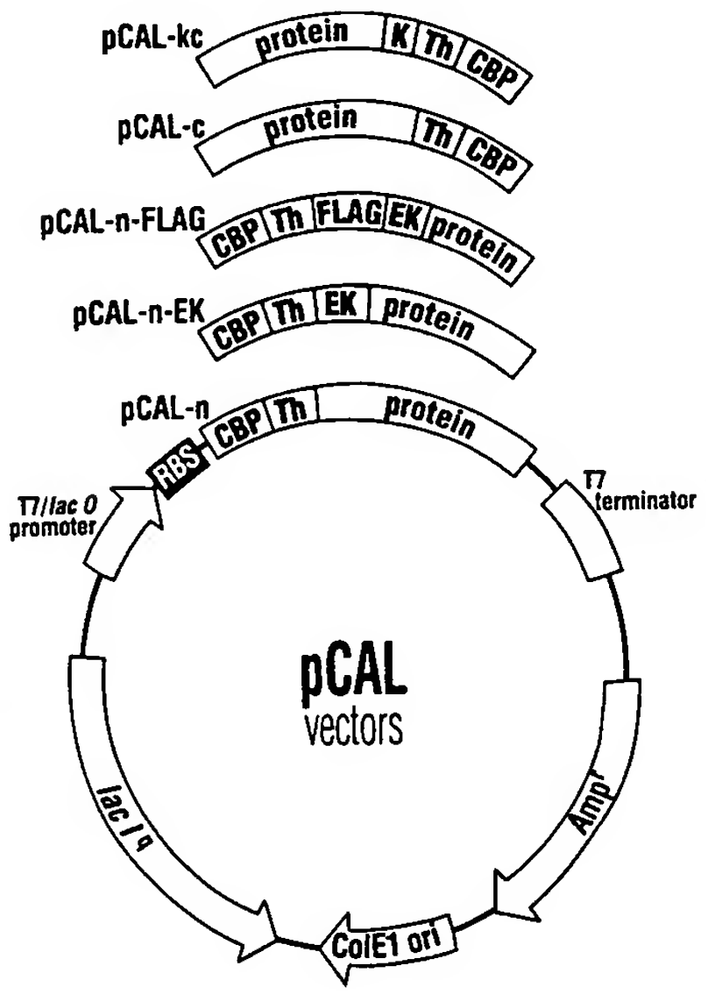


Expression of AT-Rich Genes
Cultures of BL21(DE3) or BL21-CodonPlus(DE3)-RIL cells that contained expression vectors with one of three different proteins (A, B, and C) were induced at log phase growth with IPTG. Cell lysates were separated by SDS-PAGE and visualized by staining with Coomassie® Blue dye.

BL21 Competent Cells

BL21-CodonPlus® Competent Cells (10 x 0.1 ml cells, pUC18 DNA control plasmid)		
BL21-CodonPlus-RIL	• For extremely toxic proteins, use with lambda CE6 under the control of T7 RNA polymerase • Use with non-T7 polymerase-based systems • 1x10⁷ cfu/µg of supercoiled DNA	#230240
BL21-CodonPlus(DE3)-RIL	• Encodes T7 RNA polymerase under control of the *lacUV5* promoter for easy protein expression • Use with pET or pCAL vectors • 1x10⁷ cfu/µg of supercoiled DNA	#230245
BL21-CodonPlus-RP	• For extremely toxic proteins, use with lambda CE6 under the control of T7 RNA polymerase • Use with non-T7 polymerase-based systems • 1x10⁷ cfu/µg of supercoiled DNA	#230250
BL21-CodonPlus(DE3)-RP	• Encodes T7 RNA polymerase under the control of the *lacUV5* promoter for easy protein expression • Use with pET or pCAL vectors • 1x10⁷ cfu/µg of supercoiled DNA	#230255
BL21-CodonPlus(DE3)-RIL-X	• Produce methionine-labeled proteins for structural analysis by X-ray crystallography • 1x10⁷ cfu/µg of supercoiled DNA	#230265
BL21-CodonPlus(DE3)-RP-X		#230275
BL21-Gold Competent Cells (10 x 0.1 ml cells, pUC18 DNA control plasmid)		
BL21-Gold	• For extremely toxic proteins, use with lambda CE6 under the control of T7 RNA polymerase • Use with non-T7 polymerase-based systems • 1x10⁸ cfu/µg of supercoiled DNA	#230130
BL21-Gold (DE3)	• For non-toxic proteins, encodes T7 RNA polymerase under the control of the *lacUV5* promoter • Use with pET or pCAL vectors • 1x10⁸ cfu/µg of supercoiled DNA	#230132
BL21-Gold(DE3)pLysS	• For both toxic and non-toxic proteins, codes for T7 lysozyme, a T7 RNA polymerase inhibitor • Use with pET or pCAL vectors • 1x10⁸ cfu/µg of supercoiled DNA	#230134
BL21 Competent Cells (5 x 0.2 ml cells, pUC18 DNA control plasmid)		
BL21	• For extremely toxic proteins, use with lambda CE6 under the control of T7 RNA polymerase • Use with non-T7 polymerase-based systems	#200133
BL21(DE3)	• For non-toxic proteins, encodes T7 RNA polymerase under the control of the *lacUV5* promoter • Use with pET or pCAL vectors	#200131
BL21(DE3)pLysS	• For both toxic and non-toxic proteins, pLysS plasmid codes for T7 lysozyme, a T7 RNA polymerase inhibitor • Use with pET or pCAL vectors	#200132
Lambda CE6 Induction Kit	Bacteriophage CE6, BL21 cells, LE392 host cells • For expression of toxic proteins in BL21, BL21-Gold and BL21-CodonPlus cells	#235200

Affinity® Protein Expression and Purification System



- pCAL vectors derived from T7 RNA polymerase-based pET vectors
- System consists of vectors, competent cells and resin
- Novel purification tag combines small size, gentle elution and high-affinity binding
- Native protein

APPLICATIONS
- High-level protein expression
- One-column purification

Best System for High-Level Protein Expression

The Affinity® protein expression and purification system provides a simple, gentle and effective method for high-level protein expression and purification. The system includes pCAL expression vectors containing a calmodulin-binding peptide tag, BL21(DE3) competent cells and calmodulin affinity resin (also available separately). For rapid, sensitive detection of tagged fusion proteins, use the Affinity® CBP protein detection kit.

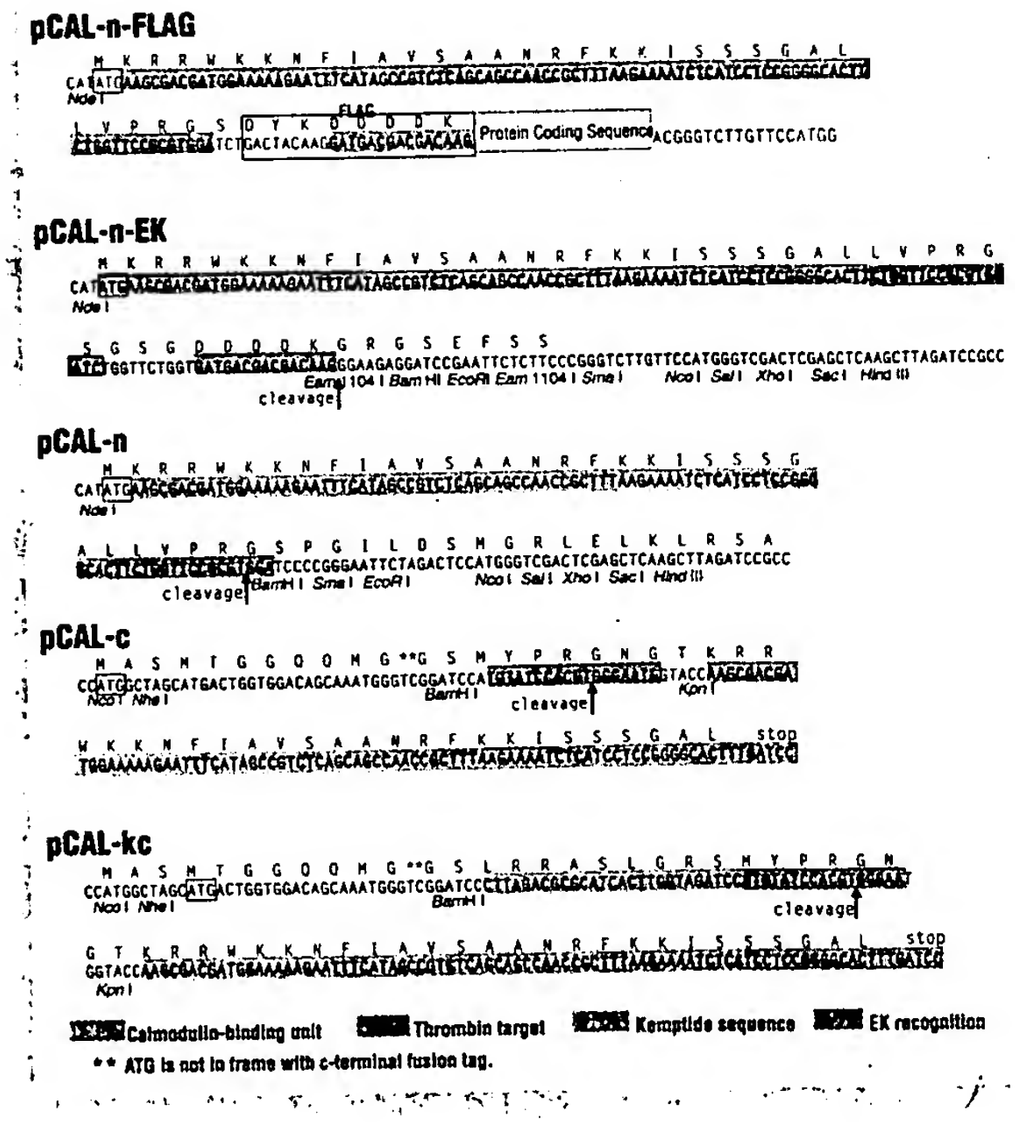
Easy Purification

The T7 RNA polymerase-based pCAL vectors express cloned proteins as fusions with the calmodulin-binding peptide (CBP) tag. The CBP-tagged proteins can be purified to near homogeneity after only one pass through calmodulin resin. The CBP tag binds to calmodulin resin in the presence of low concentrations of calcium and is eluted in the presence of 2 mM EGTA at neutral pH. It is an excellent alternative to the 6xHis affinity tag that requires harsh elution conditions. The small 4-kDa CBP tag may have less effect on the protein of interest than larger tags such as the 26-kDa GST affinity tag. Expressed proteins also include a thrombin or enterokinase** protease cleavage site, depending on the vector, for optional CBP tag removal.

pCAL Vectors

- High levels of expression
- Tight regulation
- Proteins can be expressed as C- or N-terminal fusions
- In vitro ^{32}P-labeling of expressed proteins
- pCAL-n-EK vector for LIC cloning

APPLICATIONS
• High-level protein expression • One-column purification
SELECTION
• Bacterial selection: Ampicillin
REPLICATION ORIGINS
• ColE1 origin: Plasmid origin of replication used in *E. coli*
PROMOTERS and EXPRESSION
• T7/*LacO* promoter



Highest Levels of Protein Expression

pCAL vectors are derived from the T7 RNA polymerase-based pET-11 series vectors to achieve exceptionally high levels of protein expression. All the pCAL vectors contain the T7/LacO promoter and a plasmid-borne copy of the *lacI*^{q} gene to allow tight repression in *E. coli*. In the presence of IPTG, T7 RNA polymerase is expressed in the *E. coli* BL21(DE3) or BL21(DE3)pLysS host cells. The T7 RNA polymerase binds to, and transcribes from, the T7 promoter on the pCAL vector. High-level translation utilizes the strong T7 gene 10 ribosome-binding site. Transcription and translation are highly effective and after only a few hours, the target protein may constitute the most abundant cellular protein. Using the pCAL vectors, recombinant proteins can be expressed as C- or N-terminal fusion proteins to the CBP affinity tag. The CBP portion of the expressed proteins may be labeled in vitro with cyclic AMP-dependent protein kinase and γ-^{32}P. The presence of the kemptide sequence expressed from the pCAL-kc vector allows in vitro protein labeling in experiments where the CBP tag will be removed.

Native Protein Expression

The pCAL-n-FLAG vector was designed to provide high-level protein expression, one-column purification and complete removal of the purification tag to yield purified native protein. Ligation-independent-cloning (LIC) is used to create direct cloning junctions between the gene of interest and the recognition target for the enterokinase protease (EK). Since EK cleaves at the C-terminus of its recognition site, cleavage of the fusion protein produces the native peptide (or protein) sequence, without any extra amino acids.

Affinity® LIC kits contain the pCAL-n-FLAG vector prepared for LIC cloning and reagents for LIC preparation of your PCR product. The cloning and protein purification kit also includes BL21(DE3) competent cells, calmodulin resin, EGTA for protein purification and recombinant EK for cleavage of the purification tag.

REFERENCES

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3. Means, A.R., et al. (1991) In *Cellular Calcium, A Practical Approach* (J.G. McCormack and P.H. Cobbold, eds.). IRL Press, Oxford.
4. Simcox, T.G., et al. (1995) *Strategies* 8: 40-43.
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6. Weiner, M.P., et al. (1994) *Strategies* 7: 41-43.
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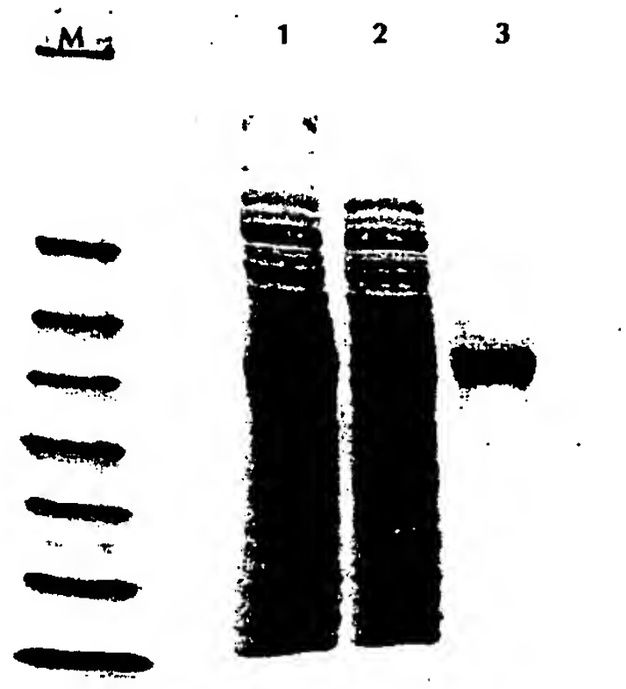
8. Lowenstein, E.J. (1992) *Cell* 70: 431-442.

* See license reference 6 on page 324.
** See license reference 7 on page 324.



Affinity Purification
The JNK gene was cloned into the pCAL-n vector and transformed into BL21(DE3) pLysS cells. Cultures were induced with IPTG and lysed. Lane 1: Input lysate, lane 2: Flowthrough lysate, lane 3: Eluate.

Affinity® Protein Expression and Purification System

CONTENTS

Affinity® Protein Expression and Purification Kits

with pCAL-n Vector	20 µg pCAL-n vector (1.0 µg/µl) 5 x 0.2-ml BL21(DE3) cells, thrombin, Calmodulin Affinity Resin, EGTA (100 mM)	• Includes most reagents for protein expression and purification • Includes pCAL-n vector	#204302
with pCAL-c Vector	20 µg pCAL-c vector (1.0 µg/µl) 5 x 0.2-ml BL21(DE3) cells, thrombin, Calmodulin Affinity Resin, EGTA (100 mM)	• Includes most reagents for protein expression and purification • Includes pCAL-c vector	#204301
with pCAL-kc Vector	20 µg pCAL-kc vector (1.0 µg/µl) 5 x 0.2-ml BL21(DE3) cells, thrombin, Calmodulin Affinity Resin, EGTA (100 mM)	• Includes most reagents for protein expression and purification • Includes pCAL-kc vector	#204300
Recombinant Enterokinase	50 U	• Includes STI agarose	#251700

Affinity® Protein Expression Vectors

pCAL-n-EK Vector	20 µg pCAL-n-EK vector *E. coli* XL1-Blue strain	• Contains enterokinase cleavage site • Derived from pET-11a • Recombinant proteins expressed as N-terminal fusion to the CBP affinity tag	#214310
pCAL-n Vector	20 µg pCAL-n vector *E. coli* XL1-Blue strain	• Derived from pET-11a • Recombinant proteins expressed as N-terminal fusion to the CBP affinity tag	#214302
pCAL-c Vector	20 µg pCAL-c vector *E. coli* XL1-Blue strain	• Derived from pET-11d • Recombinant proteins expressed as C-terminal fusion to the CBP affinity tag • Thrombin-cleavage site is immediately downstream of *Bam*H I cloning site	#214301
pCAL-kc Vector	20 µg pCAL-kc vector *E. coli* XL1-Blue strain	• Derived from pET-11d • 9-amino-acid kemptide sequence is between *Bam*H I and thrombin-cleavage site • Kemptide sequence allows proteins to be labeled with PKA and γ-^{32}P where the CBP tag has been removed	#214300
pCAL-n-FLAG Vector	20 µg pCAL-n-FLAG vector *E. coli* XL1-Blue strain	• Derived from pET-11a • Recombinant proteins expressed as N-terminal fusion to the CBP affinity tag which is followed by the Thrombin target, the FLAG epitope tag and the Enterokinase target	#214311

Calmodulin Affinity Resin

	10 ml	• Available in the Affinity system kits or separately	#214303

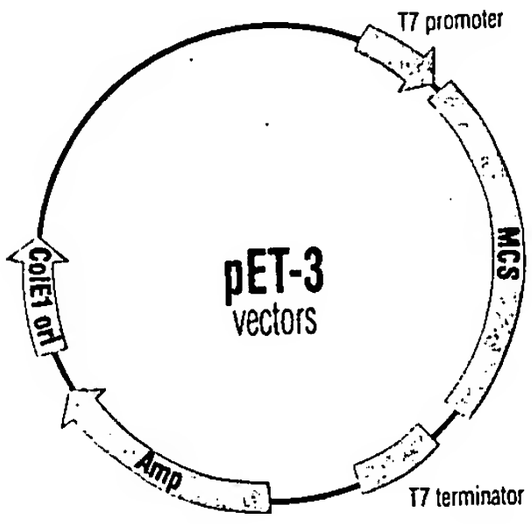
Affinity® CBP Fusion Detection Kit

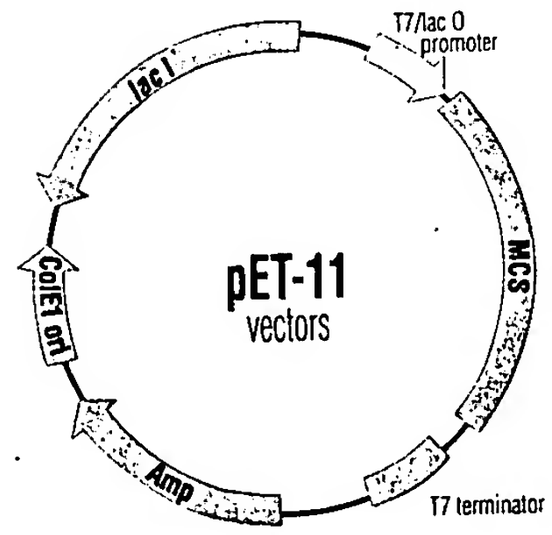
	Biotinylated calmodulin Nitroblue tetrazolium (NBT) BCIP, Tween® 20 Streptavidin alkaline phosphatase	• Rapid, sensitive detection of CBP-tagged proteins • Tagged proteins can be easily detected on a western blot • No need for specific antibodies	#200370

Tween® is a registered trademark of ICI America, Inc.

pET Expression Systems

- Tightly controlled T7 RNA polymerase-based gene expression in *E. coli*
- High-level expression upon induction
- Convenient cloning sites for optimal expression of cloned protein-coding sequence
- BL21(DE3) and BL21(DE3)pLysS competent cells are provided





APPLICATIONS
• Protein expression
SELECTION
• Bacterial selection: Ampicillin
REPLICATION ORIGINS
• ColE1 origin: Plasmid origin of replication used in *E. coli*
PROMOTERS and EXPRESSION
• pET-3 vector: T7 promoter, pET-11 vector: T7/lac O promoter

Protein Expression

The pET *E. coli* expression system* is a widely used in vivo bacterial expression system due to the strong selectivity of the bacteriophage T7 RNA polymerase for its cognate promoter sequences, the high level of activity of the polymerase and the high efficiency of translation mediated by the T7 gene *10* translation initiation signals.[1,2]

Promoter/Transcription/Expression

In the pET system, protein-coding sequence of interest is cloned downstream of the T7 promoter and gene *10* ribosome binding site, then transformed into *E. coli*. Protein expression is achieved either by IPTG induction of a chromosomally integrated cassette in which the T7 RNA polymerase is expressed from the *lacUV5* promoter (BL21(DE3) cells), or by infection with the polymerase-expressing bacteriophage lambda CE6 (BL21 cells).[3] The specificity of the T7 promoter causes extremely low basal expression of cloned target genes in strains lacking a source of T7 RNA polymerase, but upon induction, the highly active polymerase produces an abundant number of transcripts. This phenomenon plus high-efficiency translation result in such high expression levels, and after only a few hours, the target protein may constitute the majority of the cellular protein.

pET-3 Versus pET-11 Vectors

To achieve more stringent control of expression, the *lac* operator has been inserted between the T7 promoter and translation initiation sequences in the pET-11 vectors. This results in repression when IPTG is absent. Addition of IPTG causes de-repression of the T7 promoter and induction of T7 polymerase from the *lacUV5* promoter in the DE3-containing strains. The *lacI*^{q} gene is included in the pET-11 plasmids to provide adequate levels of LacI repressor protein to shut off T7 polymerase gene expression as well as T7 promoter transcription.

For high-level expression with one-column purification, see the Affinity® protein expression and purification system on previous page 103.

pET-3a, 11a

fMetAlaSerMetThrGlyGlyGlnGlnMetGlyArgGlySerGlyCysENO
GAAGGAGATATACATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGCGGATCCGGCTGCTAA...
RBS *Nde* I, *Nhe* I* *Bam*H I

pET-3b, 11b

fMetAlaSerMetThrGlyGlyGlnGlnMetGlyArgAspProAlaAla...
GAAGGAGATATACATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCCGGCTGCTAA...
RBS *Nde* I, *Nhe* I* *Bam*H I

pET-3c, 11c

fMetAlaSerMetThrGlyGlyGlnGlnMetGlyArgIleArgLeuLeu...
GAAGGAGATATACATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGATCCGGCTGCTAA...
RBS *Nde* I, *Nhe* I* *Bam*H I

pET-3d, 11d

fMetAlaSerMetThrGlyGlyGlnGlnMetGlyArgIleArgLeuLeu...
GAAGGAGATATACCATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGATCCGGCTGCTAA...
RBS *Nco* I, *Nhe* I* *Bam*H I

* *Nhe* I site is not unique in pET-3 vectors

REFERENCES
1. Weiner, M.P., et al. (1994) *Strategies* 7: 41-43.
2. Studier, F.W., et al. (1990) *Methods Enzymol.* 185: 60-89.
3. Studier, F.W. and Moffatt, B.A. (1986) *J. Mol. Biol.* 189: 113-130.
4. Phillips, T.A., VanBogelen, R.A. and Neidhardt, F.C. (1984) *J. Bacteriol.* 159: 283-287.
5. Borck, K., et al. (1976) *Mol. Gen. Genet.* 146: 199.

* See license reference 6 on page 324.

pET Expression Systems

	CONTENTS		
pET-3 Vector Series	20 µg each of pET-3a, 3b, 3c and 3d vectors 5 x 0.2-ml BL21(DE3) cells 5 x 0.2-ml BL21(DE3)pLysS cells	• pET-3 vectors contain T7 promoter	#211621
pET-11 Vector Series	20 µg each of pET-11a, 11b, 11c and 11d vectors 5 x 0.2-ml BL21(DE3) cells 5 x 0.2-ml BL21(DE3)pLysS cells	• pET-11 vectors contain T7/*lacO* promoter and *lacI*^{q} gene	#211623
pET Vectors			
pET 3a, 3b, 3c and 3d	20 µg each of pET-3a, 3b, 3c and 3d vectors	• Contain T7 promoter	#211521
pET 11a, 11b, 11c and 11d	20 µg each of pET-11a, 11b, 11c and 11d vectors	• Contain T7/*lacO* promoter and *lacI* gene	#211523

VariFlex™ Bacterial Protein Expression System

INSTRUCTION MANUAL

Catalog #240170 (N-terminal Q Vector)
#240174 (C-terminal SBP Vector)
#240182 (C-terminal Q Vector)
#240162 (N-terminal SBP Vector Set)
#240164 (N-terminal SBP-SET Vector Set)
#240166 (N-terminal SBP-SET-Q Vector Set)
#240168 (N-terminal SET-Q Vector Set)
#240172 (N-terminal SET Vector Set)
#240176 (C-terminal SBP-SET Vector Set)
#240178 (C-terminal SBP-SET-Q Vector Set)
#240180 (C-terminal SET-Q Vector Set)
#240184 (C-terminal SET Vector Set)
#240188 (N-terminal SBP-Q Vector Set)
#240190 (C-terminal SBP-Q Vector Set)
#240163 (N-terminal SBP Vector and Purification Kit)
#240165 (N-terminal SBP-SET Vector and Purification Kit)
#240175 (C-terminal SBP Vector and Purification Kit)
#240177 (C-terminal SBP-SET Vector and Purification Kit)
#240169 (N-terminal SET-Q Vector and Detection Kit)
#240171 (N-terminal Q Vector and Detection Kit)
#240181 (C-terminal SET-Q Vector and Detection Kit)
#240183 (C-terminal Q Vector and Detection Kit)
#240167 (N-terminal SBP-SET-Q Vector, Purification, and Detection Kit)
#240179 (C-terminal SBP-SET-Q Vector, Purification, and Detection Kit)
#240189 (N-terminal SBP-Q Vector, Purification, and Detection Kit)
#240191 (C-terminal SBP-Q Vector, Purification, and Detection Kit)

Revision #104002a

For In Vitro Use Only

240162-12 104002A/

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	0800 919 288	0800 919 287	0800 919 289
Germany	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 182 8232	0800 182 8231	0800 182 8234
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	0800 023 0446	+31 (0)20 312 5700	0800 023 0448
Switzerland	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 563 080	0800 563 082	0800 563 081
United Kingdom	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 917 3282	0800 917 3283	0800 917 3281

All Other Countries

Please contact your local distributor. A complete list of distributors is available at *www.stratagene.com*.

VariFlex™ Bacterial Protein Expression System

CONTENTS

Materials Provided 1
Storage Conditions 5
Additional Materials Required 5
Notices to Purchaser 6
Introduction 8
The VariFlex™ Protein Expression Vectors 10
pBEn-SBP-SET1-Qa Vector Map 11
pBEc-SBP-SET1-Q Vector Map 12
BL21 Expression Strains 17
Preparing the Vectors 19
Ligating the Insert 20
Transforming the Ligation Reactions 21
Transformation Guidelines 21
Transformation Protocol 22
Expected Transformation Results 23
Induction of the Target Protein Using IPTG 23
Affinity Purification of the SBP-tagged Protein 24
Preparing the Streptavidin Resin 24
Purifying the SBP-tagged Protein Using Streptavidin Resin 24
Protein Quantification Using the Q-tag 26
Removing the Tags with Thrombin 28
Troubleshooting 29
Preparation of Media and Reagents 30
References 32
Endnotes 32
MSDS Information 32

VariFlex™ Bacterial Protein Expression System

MATERIALS PROVIDED

VariFlex™ Q-tag Detection Kit (Catalog #240186)

Component	Quantity
Cell lysis buffer	6 ml
EA reagent	2 ml
ED control (10 nM)	180 µl
CL substrate	160 µl
CL enhancer	800 µl
CL substrate diluent	3.04 ml

- Sufficient detection reagents are provided for one hundred reactions.

BL21-Gold(DE3)LacZ⁻ Competent Cells (Catalog #230135)

Component	Concentration	Quantity
BL21-Gold(DE3)LacZ⁻ competent cells (Catalog #230135)	—	10 × 100 µl
pUC18 control vector	0.1 ng/µl	10 µl

N-terminal SBP Vector Set (Catalog #240162)

Component	Concentration	Quantity
pBEn-SBPa vector	1 µg/µl	20 µg
pBEn-SBPb vector	1 µg/µl	20 µg
pBEn-SBPc vector	1 µg/µl	20 µg

N-terminal SBP Vector and Purification Kit (Catalog #240163)

Component	Concentration	Quantity
N-terminal SBP Vector Set (Catalog #240162)	1 µg/µl	20 µg of each vector
Streptavidin resin	—	1.25 ml

N-terminal SBP-SET Vector Set (Catalog #240164)

Component	Concentration	Quantity
pBEn-SBP-SET1a vector	1 µg/µl	20 µg
pBEn-SBP-SET1b vector	1 µg/µl	20 µg
pBEn-SBP-SET1c vector	1 µg/µl	20 µg
pBEn-SBP-SET2a vector	1 µg/µl	20 µg
pBEn-SBP-SET2b vector	1 µg/µl	20 µg
pBEn-SBP-SET2c vector	1 µg/µl	20 µg
pBEn-SBP-SET3a vector	1 µg/µl	20 µg
pBEn-SBP-SET3b vector	1 µg/µl	20 µg
pBEn-SBP-SET3c vector	1 µg/µl	20 µg

Revision #104002a

N-terminal SBP-SET Vector and Purification Kit (Catalog #240165)

Component	Concentration	Quantity
N-terminal SBP-SET Vector Set (Catalog #240164)	1 µg/µl	20 µg of each vector
Streptavidin resin	—	1.25 ml

N-terminal SBP-SET-Q Vector Set (Catalog #240166)

Component	Concentration	Quantity
pBEn-SBP-SET1-Qa vector	1 µg/µl	20 µg
pBEn-SBP-SET1-Qb vector	1 µg/µl	20 µg
pBEn-SBP-SET1-Qc vector	1 µg/µl	20 µg
pBEn-SBP-SET2-Qa vector	1 µg/µl	20 µg
pBEn-SBP-SET2-Qb vector	1 µg/µl	20 µg
pBEn-SBP-SET2-Qc vector	1 µg/µl	20 µg
pBEn-SBP-SET3-Qa vector	1 µg/µl	20 µg
pBEn-SBP-SET3-Qb vector	1 µg/µl	20 µg
pBEn-SBP-SET3-Qc vector	1 µg/µl	20 µg

N-terminal SBP-SET-Q Vector, Purification, and Detection Kit (Catalog #240167)

Component	Concentration	Quantity
N-terminal SBP-SET-Q Vector Set (Catalog # 240166)	1 µg/µl	20 µg of each vector
Streptavidin resin	—	1.25 ml
VariFlex™ Q-tag detection kit (Catalog #240186)	—	1 kit
BL21-Gold(DE3)LacZ^{-} competent cells (Catalog #230135)	—	10 × 100 µl cells

N-terminal SET-Q Vector Set (Catalog #240168)

Component	Concentration	Quantity
pBEn-SET1-Q vector	1 µg/µl	20 µg
pBEn-SET2-Q vector	1 µg/µl	20 µg
pBEn-SET3-Q vector	1 µg/µl	20 µg

N-terminal SET-Q Vector and Detection Kit (Catalog #240169)

Component	Concentration	Quantity
N-terminal SET-Q Vector Set (Catalog #240168)	1 µg/µl	20 µg of each vector
VariFlex™ Q-tag detection kit (Catalog #240186)	—	1 kit
BL21-Gold(DE3)LacZ^{-} competent cells (Catalog #230135)	—	10 × 100 µl cells

N-terminal SBP-Q Vector (Catalog #240188)

Component	Concentration	Quantity
pBEn-SBP-Q vector	1 µg/µl	20 µg

N-terminal SBP-Q Vector, Purification, and Detection Kit (Catalog #240189)

Component	Concentration	Quantity
N-terminal SBP-Q Vector (Catalog #240188)	—	20 µg
Streptavidin resin	—	1.25 ml
VariFlex™ Q-tag detection kit (Catalog #240186)	—	1 kit
BL21-Gold(DE3)LacZ^{-} competent cells (Catalog #230135)	—	10 × 100 µl cells

N-terminal Q Vector (Catalog #240170)

Component	Concentration	Quantity
pBEn-Q vector	1 µg/µl	20 µg

N-terminal Q Vector and Detection Kit (Catalog #240171)

Component	Concentration	Quantity
N-terminal Q Vector (Catalog #240170)	—	20 µg
VariFlex™ Q-tag detection kit (Catalog #240186)	—	1 kit
BL21-Gold(DE3)LacZ^{-} competent cells (Catalog #230135)	—	10 × 100 µl cells

N-terminal SET Vector Set (Catalog #240172)

Component	Concentration	Quantity
pBEn-SET1a vector	1 µg/µl	20 µg
pBEn-SET1b vector	1 µg/µl	20 µg
pBEn-SET1c vector	1 µg/µl	20 µg
pBEn-SET2a vector	1 µg/µl	20 µg
pBEn-SET2b vector	1 µg/µl	20 µg
pBEn-SET2c vector	1 µg/µl	20 µg
pBEn-SET3a vector	1 µg/µl	20 µg
pBEn-SET3b vector	1 µg/µl	20 µg
pBEn-SET3c vector	1 µg/µl	20 µg

C-terminal SBP Vector (Catalog #240174)

Component	Concentration	Quantity
pBEc-SBP vector	1 µg/µl	20 µg

C-terminal SBP Vector and Purification Kit (Catalog #240175)

Component	Concentration	Quantity
C-terminal SBP Vector (Catalog #240174)	—	20 µg
Streptavidin resin	—	1.25 ml

C-terminal SBP-SET Vector Set (Catalog #240176)

Component	Concentration	Quantity
pBEc-SBP-SET1 vector	1 µg/µl	20 µg
pBEc-SBP-SET2 vector	1 µg/µl	20 µg
pBEc-SBP-SET3 vector	1 µg/µl	20 µg

C-terminal SBP-SET Vector and Purification Kit (Catalog #240177)

Component	Concentration	Quantity
C-terminal SBP-SET Vector Set (Catalog #240176)	1 µg/µl	20 µg of each vector
Streptavidin resin	—	1.25 ml

C-terminal SBP-SET-Q Vector Set (Catalog #240178)

Component	Concentration	Quantity
pBEc-SBP-SET1-Q vector	1 µg/µl	20 µg
pBEc-SBP-SET2-Q vector	1 µg/µl	20 µg
pBEc-SBP-SET3-Q vector	1 µg/µl	20 µg

C-terminal SBP-SET-Q Vector, Purification, and Detection Kit (Catalog #240179)

Component	Concentration	Quantity
C-terminal SBP-SET-Q Vector Set (Catalog #240178)	1 µg/µl	20 µg of each vector
Streptavidin resin	—	1.25 ml
Variflex™ Q-tag detection kit (Catalog # 240186)	—	1 kit
BL21-Gold(DE3)LacZ^{-} competent cells (Catalog #230135)	—	10 × 100 µl cells

C-terminal SET-Q Vector Set (Catalog #240180)

Component	Concentration	Quantity
pBEc-SET1-Q vector	1 µg/µl	20 µg
pBEc-SET2-Q vector	1 µg/µl	20 µg
pBEc-SET3-Q vector	1 µg/µl	20 µg

C-terminal SET-Q Vector and Detection Kit (Catalog #240181)

Component	Concentration	Quantity
C-terminal SET-Q Vector Set (Catalog #240180)	1 µg/µl	20 µg of each vector
VariFlex™ Q-tag detection kit (Catalog #240186)	—	1 kit
BL21-Gold(DE3)LacZ^{-} competent cells (Catalog #230135)	—	10 × 100 µl cells

C-terminal Q Vector (Catalog #240182)

Component	Concentration	Quantity
pBEc-Q vector	1 µg/µl	20 µg

C-terminal Q Vector and Detection Kit (Catalog #240183)

Component	Concentration	Quantity
C-terminal Q Vector (Catalog # 240182)	1 µg/µl	20 µg
VariFlex™ Q-tag detection kit (Catalog #240186)	—	1 kit
BL21-Gold(DE3)LacZ^{-} competent cells (Catalog #230135)	—	10 × 100 µl cells

C-terminal SET Vector Set (Catalog #240184)

Component	Concentration	Quantity
pBEc-SET1 vector	1 µg/µl	20 µg
pBEc-SET2 vector	1 µg/µl	20 µg
pBEc-SET3 vector	1 µg/µl	20 µg

C-terminal SBP-Q Vector (Catalog #240190)

Component	Concentration	Quantity
pBEc-SBP-Q vector	1 µg/µl	20 µg

C-terminal SBP-Q Vector, Purification, and Detection Kit (Catalog #240191)

Component	Concentration	Quantity
C-terminal SBP-Q Vector (Catalog #240190)	—	20 µg
Streptavidin resin	—	1.25 ml
VariFlex™ Q-tag detection kit (Catalog #240186)	—	1 kit
BL21-Gold(DE3)LacZ^{-} competent cells (Catalog #230135)	—	10 × 100 µl cells

Storage Conditions

BL21-Gold(DE3)LacZ⁻ competent cells: Store the cells immediately at –80°C.
Do not place the cells in liquid nitrogen.
All Other Components: –20°C

Additional Materials Required

14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
BL21-Gold (DE3) competent cells
Ligase buffer[§]
plate reader
protease inhibitors
T4 DNA ligase
TE buffer[§]
Thrombin

§ See *Preparation of Media and Reagents*.

NOTICES TO PURCHASER

Q-tag Detection Kit License Agreement

Detection technology licensed from DiscoveRx Corporation (Fremont, CA). For more information, please visit *www.DiscoveRx.com.*

SBP Tag License Agreement

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INTRODUCTION

Stratagene's VariFlex™ bacterial protein expression system is a series of pET-based vectors that offer solutions to challenges in protein expression and enhance the utility of *E. coli* as an expression host. Available tags include three different solubility enhancement tags (SETs) which are designed to increase protein solubility, the streptavidin binding peptide (SBP) purification tag, and a tag that allows for rapid soluble protein quantification (Q-tag). Figure 1 contains the amino acid sequences of each tag. The VariFlex vectors are available with one, two, or three tags in various combinations.

One of the most difficult problems in expressing eukaryotic genes in bacterial systems is the lack of solubility of the expression product. Often times, expression in a more time-consuming, lower-yielding, expensive host such as yeast, insect, or mammalian cells is necessary. To address this issue, the VariFlex SET tags increase the solubility of many problem proteins in *E. coli*. Although the mechanism by which the SET tags improve solubility has not yet been confirmed, the tags are thought to enhance solubility of the fusion peptide by providing a net negative charge, which is thought to prevent aggregation and provide more time for correct protein folding in vivo.[1] The SET tags are based on the C-terminal portion of the T7 phage gene 10B sequence (T7B) which has a net charge of –6. The SET1 tag is the wild-type T7B sequence, while the SET2 and SET3 tags are mutants of T7B that further increase the net negative charge to –12 and –18 respectively. Since every protein is unique, the optimal SET tag needs to be determined empirically for each protein of interest. Stratagene therefore offers the SET-tagged vectors as complete sets, where vectors containing each of the three SET tag variants are provided.

In addition to the SET tags, Stratagene offers vectors containing the SBP tag, which provides efficient purification of the protein of interest. The SBP tag, a synthetic sequence isolated from a random peptide library, has a high affinity for streptavidin resin (~2×10^{-9} M), and can be effectively eluted with biotin.[2,3] The SBP tag has a low positive net charge (+1), making it an ideal purification tag when combined with the SET tags, since its affect on the SET tag negative charge is minimal.

The ability to rapidly quantify the protein of interest in a given lysate makes high throughput protein expression experiments less time consuming by eliminating the need for tedious PAGE gel quantification. Thus, the third feature of the VariFlex system is the Q-tag and the Q-tag detection method, which determine whether the protein of interest exists in the soluble fraction and how much soluble protein is produced. The Q-tag assay is based on in vitro α-complementation of the β-galactosidase protein, where the Q-tag encodes the α-fragment of β-galactosidase in a *lacZ* deficient BL21 protein expression *E. coli* host. Q-tagged proteins in cell lysates are quantified in vitro after the addition of the complementing β-gal fragment and a chemiluminescent detection substrate. When the Q-tag and the inactive complementing fragment interact, β-gal enzyme activity is restored, and the detection substrate gives a read-out of enzyme activity (see Figure 2).

SET1 tag	MDPEEASVTSTEETLTPAQEAARTRAANKARKEAELAAATAEQ
SET2 tag	MDPEEASVTSTEETLTPAQEAAETEAANKARKEAELEAETAEQ * * * *
SET3 tag	MDPEEASVTSTEETLTPAQEAAETEAANKAEEEAELEAETAEQ * * ** * *
SBP tag	MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPSGGCKLG
Q-tag	MSSNSLAVVLQRRDWENPGVTQLNRLAAHPPFASWRNSEEARTDRPSQQLRSLNGE

FIGURE 1 Amino acid sequences of the solubility enhancement tags (SET1, SET2, and SET3), the streptavidin binding peptide (SBP), and the protein quantification tag (Q-tag). The asterisks indicate mutations present in the variants of the SET tag.

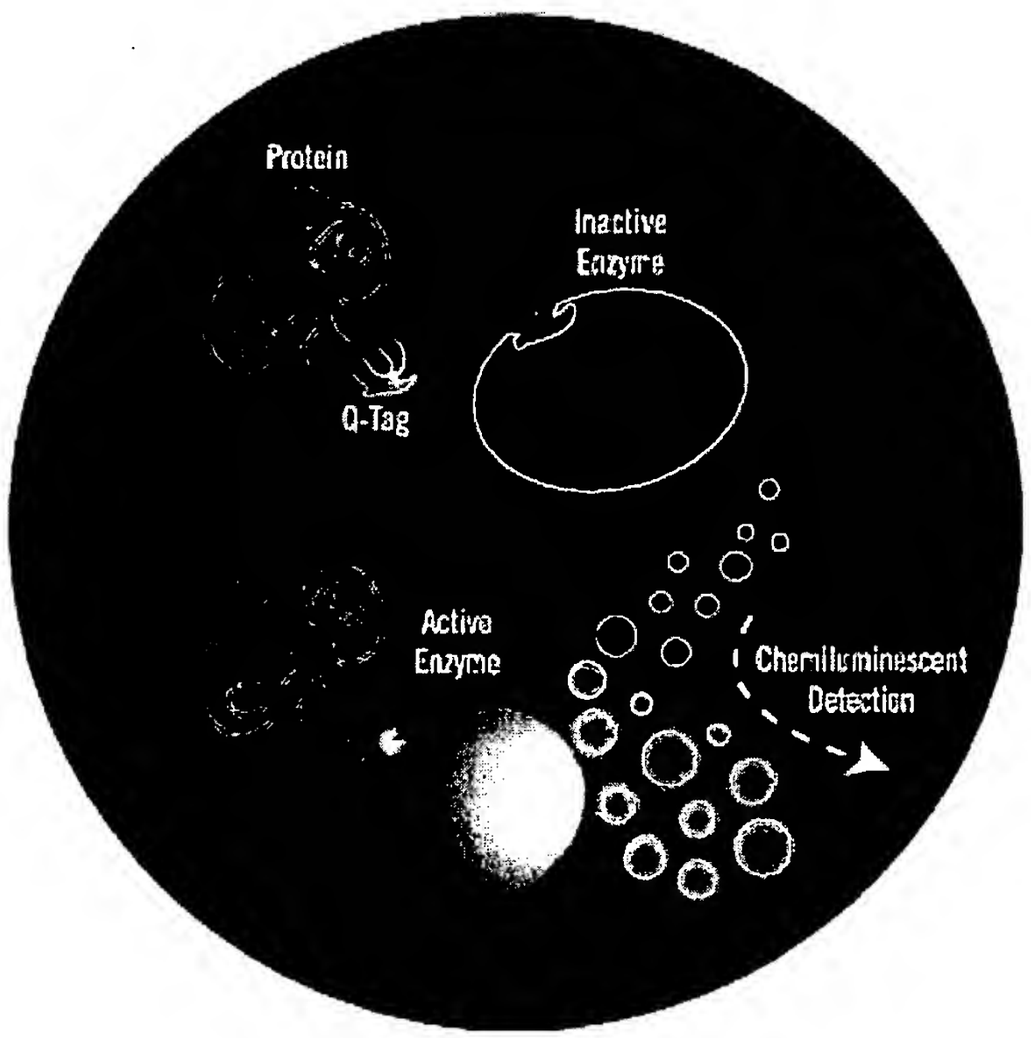


FIGURE 2 The Q-tag peptide encodes for the α portion of the β-galactosidase protein. In the Q-tag assay, a non-functional fragment of the β-galactosidase protein and a chemiluminescent detection substrate are added to the cell lysate, which contains the Q-tagged protein of interest. When the Q-tag and the inactive fragment interact, enzyme activity is restored, and the detection substrate gives a read-out of enzyme activity.

THE VARIFLEX™ PROTEIN EXPRESSION VECTORS

The VariFlex protein expression vectors are derived from the pET-11 vector series (see Figure 2). The vectors are engineered to take advantage of the features of the bacteriophage T7 gene *10* promoter and leader sequence that allow high selectivity of the promoter by T7 RNA polymerase, tight repression in the uninduced state, and high-level expression upon induction.[4,5] The VariFlex vectors use the T7 *lac* promoter configuration and carry a copy of the *lacI* gene to mediate this tight repression.

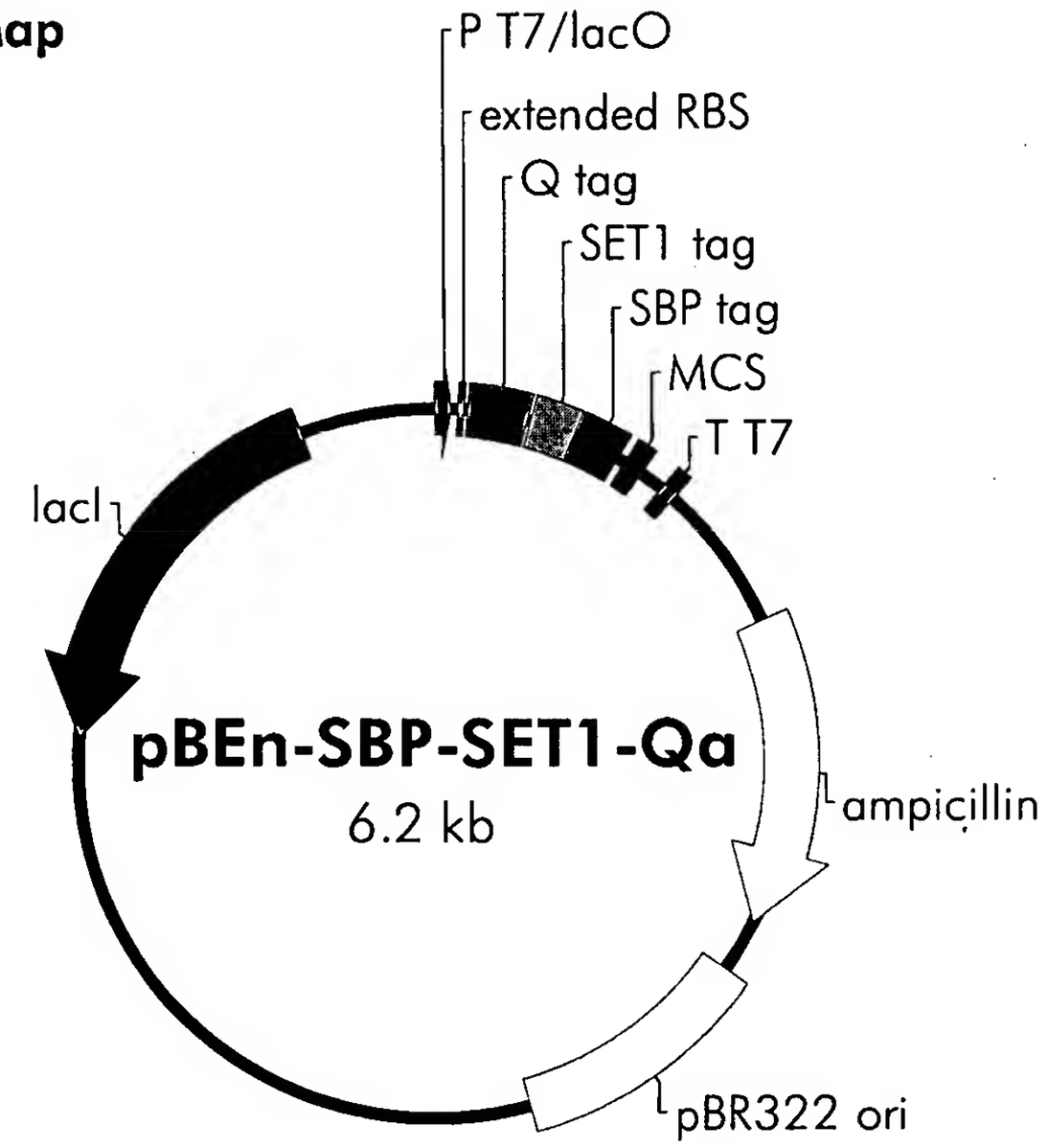
Each VariFlex vector carries one to three tags in different combinations, providing flexibility depending on the desired applications. These tags include the solubility enhancement tags 1–3, the streptavidin binding peptide for protein purification, and a quantitative tag to quantitate the amount of soluble protein in a given lysate. The tags are available as fusions to either the N- or C-terminus of the protein of interest, and most N-terminal vectors are provided in all three reading frames. A thrombin protease cleavage site exists between the tag(s) and the cloning sites so the tags can be easily removed for further protein analysis.

The pBEn vectors are based on the pET-11a vector and contain the tag coding sequence(s) inserted upstream of a multiple cloning site (MCS) to allow for fusion of the tag(s) at the N-terminus of the cloned protein-coding sequence. The efficient translation of the tags in *E. coli* ensures that fusion proteins containing the tags at the N terminus will be consistently expressed at high levels. The recognition sequence for thrombin is inserted between the tag coding sequences and the MCS. Digestion of purified fusion protein with thrombin occurs between the arginine and glycine residues within the thrombin recognition sequence.

The pBEc vectors are based on the pET-11d vector and contain the tag coding sequence(s) inserted downstream of the cloning site to allow for fusion of the tag(s) at the C-terminus of the cloned protein-coding sequence. Inserts are cloned between the *Nco* I site, which contains an ATG positioned for optimal translation from the T7 gene *10* ribosome-binding site (RBS), and the *Bam*H I site. Alternatively, inserts can be cloned between the *Nhe* I and *Bam*H I sites. Thrombin digestion of proteins expressed from the pBEc vectors result in the retention of the four N-terminal amino acids (MYPR) from the thrombin recognition sequence.

Caution *The T7 gene 10 leader and the C-terminal fusion tags, beginning with the Gly-Ser residues encoded by the* Bam*H I restriction site, are in* ***separate frames****. Although bi-directional cloning is not recommended, if cloning into the* Bam*H I restriction site, care should be taken that the protein coding sequence of interest is fused in frame with both the T7 gene 10 leader and the C-terminal fusion tag. If cloning bi-directionally into* Nco *I or* Nhe *I, the inserted amino acid sequence should be in frame with the C-terminal fusion tag beginning with the Gly-Ser residues encoded by the* Bam*H I site.*

pBEn-SBP-SET1-Qa Vector Map



pBEn-SBP-SET1-Qa Multiple Cloning Site Region

sequence shown (497–607)

thrombin cleavage ▼

...CAG CGG GAG CCC TCC GGC GGC TGC AAG CTG GGC CTG CTG GTT CCG CGT GGA TCT...

Q R E P S G G C K L G L L V P R G S

streptavidin binding peptide — thrombin target

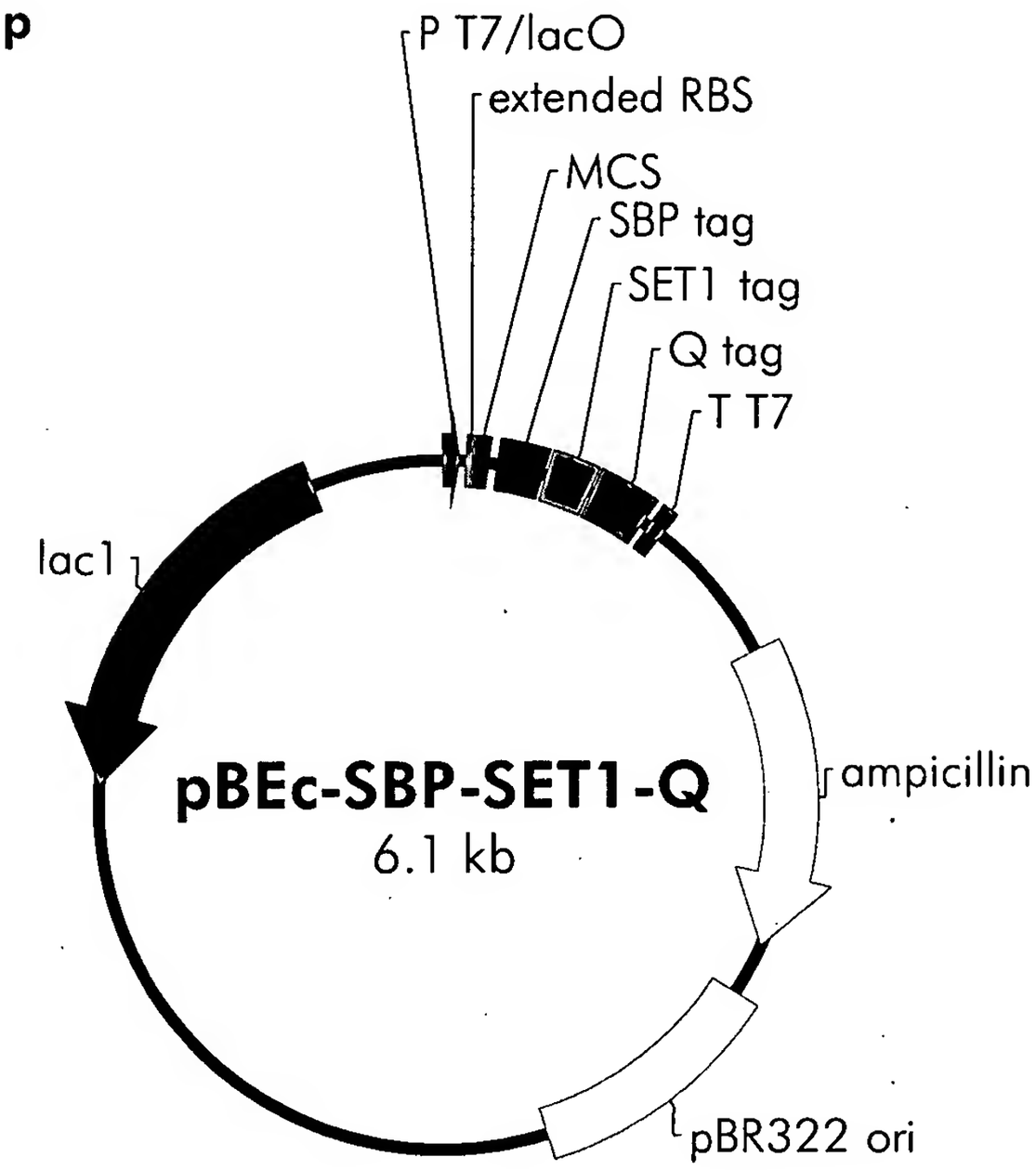
BamH I EcoR I Sma I Nco I Sal I Xho I Hind III

...GGA TCC GAA TTC TCT TCC CGG GTC TTG TTC CAT GGG TCG ACT CGA GCT CAA GCT TAG

STOP

FIGURE 3 Vector map and multiple cloning region of the pBEn-SBP-SET1-Qa bacterial protein expression vector. See Table I for vector feature locations. This figure is intended to be a general representation of the N-terminal vectors. Other vectors may include different tag combinations, reading frames, and restriction sites. For specific vector information, please refer to *http://www.stratagene.com/lit/vector.aspx*.

pBEc-SBP-SET1-Q Vector Map



pBEc-SBP-SET1-Q Multiple Cloning Site Region

sequence shown (87–206)

Nco I Nhe I BamH I thrombin cleavage

CC ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT C GGA TCC ATG TAT CCA CGT GGG AAT...

M A S M T G G Q Q M G * G S M Y P R G N

START

T7 gene 10 leader peptide thrombin target

Kpn I

...GGT ACC GAC GAG AAG ACC ACC GGC TGG CGG GGC GGC CAC GTG GTG GAG GGC CTG GCC GGC

G T Q E K T T G W R G G H V V E G L A G

streptavidin-binding peptide

*ATG is not in frame with the C-terminal fusion tags.

FIGURE 4 Vector map and multiple cloning region of the pBEc-SBP-SET1-Qa bacterial protein expression vector. See Table I for vector feature locations. This figure is intended to be a general representation of the C-terminal vectors. Other vectors may include different tag combinations, reading frames, and restriction sites. For specific vector information, please refer to http://www.stratagene.com/lit/vector.aspx.

TABLE I

Features of the Bacterial Protein Expression Vectors

	T7 promoter with *lac* operon	extended ribosome binding site	protein quantification tag (Q-tag)	solubility enhancement tag 1 (SET1 tag)	solubility enhancement tag 2 (SET2 tag)	solubility enhancement tag 3 (SET3 tag)	streptavidin binding peptide (SBP tag)	multiple cloning site	T7 terminator	ampicillin resistance (*bla*) ORF	pBR322 origin of replication	*lacI* repressor ORF
pBEn-SBPa	1–44	64–80	—	—	—	—	92–223	245–299	369–411	832–1689	1840–2507	4387–5475
pBEn-SBPb	1–44	64–80	—	—	—	—	92–223	246–300	370–412	883–1690	1841–2508	4388–5476
pBEn-SBPc	1–44	64–80	—	—	—	—	92–223	247–301	371–413	834–1691	1842–2509	4389–5477
pBEn-SBP-SET1a	1–44	64–80	—	92–217	—	—	224–355	377–431	501–543	964–1821	1972–2639	4519–5607
pBEn-SBP-SET1b	1–44	64–80	—	92–217	—	—	224–355	378–432	502–544	965–1812	1973–2640	4520–5608
pBEn-SBP-SET1c	1–44	64–80	—	92–217	—	—	224–355	379–433	503–545	966–1823	1974–2641	4521–5609
pBEn-SBP-SET2a	1–44	64–80	—	—	92–217	—	224–355	377–431	501–543	964–1821	1972–2639	4519–5607
pBEn-SBP-SET2b	1–44	64–80	—	—	92–217	—	224–355	378–432	502–544	965–1822	1973–2640	5420–5608
pBEn-SBP-SET2c	1–44	64–80	—	—	92–217	—	224–355	379–433	503–545	966–1823	1947–2641	4521–5609
pBEn-SBP-SET3a	1–44	64–80	—	—	—	92–217	224–355	377–431	501–543	964–1821	1972–2639	4519–5607
pBEn-SBP-SET3b	1–44	64–80	—	—	—	92–217	224–355	378–432	502–544	965–1822	1973–2640	4520–5608

	T7 promoter with *lac* operon	extended ribosome binding site	protein quantification tag (Q-tag)	solubility enhancement tag 1 (SET1 tag)	solubility enhancement tag 2 (SET2 tag)	solubility enhancement tag 3 (SET3 tag)	streptavidin binding peptide (SBP tag)	multiple cloning site	T7 terminator	ampicillin resistance (*bla*) ORF	pBR322 origin of replication	*lacI* repressor ORF
pBEn-SBP-SET3c	1–44	64–80	—	—	—	92–217	224–355	379–433	503–545	966–1823	1974–2641	4521–5609
pBEn-SBP-SET1-Qa	1–44	64–80	92–256	266–391	—	—	398–529	551–605	675–717	1138–1995	2146–2813	4693–5781
pBEn-SBP-SET1-Qb	1–44	64–80	92–256	266–391	—	—	398–529	552–606	676–718	1139–1996	2147–2814	4694–5782
pBEn-SBP-SET1-Qc	1–44	64–80	92–256	266–391	—	—	398–529	553–607	677–719	1140–1997	2148–2815	4695–5783
pBEn-SBP-SET2-Qa	1–44	64–80	92–256	—	266–391	—	398–529	551–605	675–717	1138–1995	2146–2813	4693–5781
pBEn-SBP-SET2-Qb	1–44	64–80	92–256	—	266–391	—	398–529	552–606	676–718	1139–1996	2147–2814	4694–5782
pBEn-SBP-SET2-Qc	1–44	64–80	92–256	—	266–391	—	398–529	553–607	677–719	1140–1997	2148–2815	4695–5783
pBEn-SBP-SET3-Qa	1–44	64–80	92–256	—	—	266–391	398–529	551–605	675–717	1138–1995	2146–2813	4693–5781
pBEn-SBP-SET3-Qb	1–44	64–80	92–256	—	—	266–391	398–529	552–606	676–718	1139–1996	2147–2814	4694–5782
pBEn-SBP-SET3-Qc	1–44	64–80	92–256	—	—	266–391	398–529	553–607	677–719	1140–1997	2148–2815	4695–5783
pBEn-SBP-Q	1–44	64–80	92–256	—	—	—	278–394	416–470	540–582	1003–1860	2011–2678	4558–5646
pBEn-SET1a	1–44	64–80	—	92–217	—	—	—	239–293	363–405	826–1683	1834–2501	4381–5469
pBEn-SET1b	1–44	64–80	—	92–217	—	—	—	240–294	364–406	827–1684	1835–2502	4382–5470

	T7 promoter with *lac* operon	extended ribosome binding site	protein quantification tag (Q-tag)	solubility enhancement tag 1 (SET1 tag)	solubility enhancement tag 2 (SET2 tag)	solubility enhancement tag 3 (SET3 tag)	streptavidin binding peptide (SBP tag)	multiple cloning site	T7 terminator	ampicillin resistance (*bla*) ORF	pBR322 origin of replication	*lacI* repressor ORF
pBEn-SET1c	1–44	64–80	—	92–217	—	—	—	241–295	365–407	828□01685	1836–2503	4383–5471
pBEn-SET2a	1–44	64–80	—	—	92–217	—	—	239–293	363–405	826–1683	1834–2501	4381–5469
pBEn-SET2b	1–44	64–80	—	—	92–217	—	—	240–294	364–406	827–1684	1835–2502	4382–5470
pBEn-SET2c	1–44	64–80	—	—	92–217	—	—	241–295	365–407	828–1685	1836–2503	4383–5471
pBEn-SET3a	1–44	64–80	—	—	—	92–217	—	239–293	363–405	826–1683	1834–2501	4381–5469
pBEn-SET3b	1–44	64–80	—	—	—	92–217	—	240–294	364–406	827–1684	1835–2502	4382–5470
pBEn-SET3c	1–44	64–80	—	—	—	92–217	—	241–295	365–407	828–1685	1836–2503	4383–5471
pBEn-SET1-Q	1–44	64–80	92–256	413–467	—	—	—	413–467	537–579	1000–1857	2008–2675	4555–5643
pBEn-SET2-Q	1–44	64–80	92–256	—	266–391	—	—	413–467	537–579	1000–1857	2008–2675	4555–5643
pBEn-SET3-Q	1–44	64–80	92–256	—	—	266–391	—	413–467	537–579	1000–1857	2008–2675	4555–5643
pBEn-Q	1–44	64–80	92–256	—	—	—	—	278–332	402–444	865–1722	1873–2540	4420–5508
pBEc-SBP	2–45	65–81	—	—	—	—	153–284	87–128	296–338	759–1616	1767–2434	4314–5402
pBEc-SBP-SET1	2–45	65–81	—	294–419	—	—	153–284	87–128	440–482	903–1760	1911–2578	4469–5546

	T7 promoter with *lac* operon	extended ribosome binding site	protein quantification tag (Q-tag)	solubility enhancement tag 1 (SET1 tag)	solubility enhancement tag 2 (SET2 tag)	solubility enhancement tag 3 (SET3 tag)	streptavidin binding peptide (SBP tag)	multiple cloning site	T7 terminator	ampicillin resistance (*bla*) ORF	pBR322 origin of replication	*lacI* repressor ORF
pBEc-SBP-SET2	2–45	65–81	—	—	294–419	—	153–284	87–128	440–482	903–1760	1911–2578	4458–5546
pBEc-SBP-SET3	2–45	65–81	—	—	—	294–419	153–284	87–128	440–482	903–1760	1911–2578	4458–5546
pBEc-SBP-SET1-Q	2–45	65–81	429–593	294–419	—	—	153–284	87–128	614–656	1077–1934	2085–2752	4632–5720
pBEc-SBP-SET2-Q	2–45	65–81	429–593	—	294–419	—	153–284	87–128	614–656	1077–1934	2085–2752	4632–5720
pBEc-SBP-SET3-Q	2–45	65–81	429–593	—	—	294–419	153–284	87–128	614–656	1077–1934	2085–2752	4632–5720
pBEc-SET1-Q	2–45	65–81	288–452	153–278	—	—	—	87–128	473–515	936–1793	1944–2611	4491–5579
pBEc-SET2-Q	2–45	65–81	288–452	—	153–278	—	—	87–128	473–515	936–793	1944–2611	4491–5579
pBEc-SET3-Q	2–45	65–81	288–452	—	—	153–278	—	87–128	473–515	936–1793	1944–2611	4491–5579
pBEc-Q	2–45	65–81	156–320	—	—	—	—	87–128	341–383	804–1661	1812–2479	4359–5447
pBEc-SET1	2–45	65–81	—	153–278	—	—	—	87–128	299–341	762–1619	1770–2437	4317–5405
pBEc-SET2	2–45	65–81	—	—	153–278	—	—	87–128	299–341	762–1619	1770–2437	4317–5405
pBEc-SET3	2–45	65–81	—	—	—	153–278	—	87–128	299–341	762–1619	1770–2437	4317–5405
pBEc-SBP-Q	2–45	65–81	294–458	—	—	—	153–284	87–128	479–521	942–1799	1950–2617	4497–5585

BL21 Expression Strains

BL21 expression strains are recommended for use with the VariFlex vectors because of their compatibility with pET-derived vector features. As a general protein expression strain, the BL21 expression strain, derived from the *E. coli* B strain BL21, is superior due to its deficiency in *lon* protease as well as the *ompT* outer membrane protease that can degrade proteins during purification.[6–8] Modifications to the BL21 strain include the DE3 and Gold features. The BL21(DE3) strain[4,6] carries a lambda DE3 lysogen that has the phage 21 immunity region, the *lacI* gene, and the *lacUV5*-driven T7 RNA polymerase expression cassette. On induction with IPTG, the *lacUV5* promoter is derepressed, allowing overexpression of T7 RNA polymerase and expression of the T7-promoted target gene. The BL21-Gold-derived expression strains feature the Hte phenotype which increases the transformation efficiency of the BL21-Gold cells. In addition, the gene that encodes endonuclease I (*endA*), which rapidly degrades vector DNA isolated by most miniprep procedures, is inactivated.

Additionally, there are a variety of BL21 host strains designed to address specific protein expression problems. These problems include the toxicity of the gene product and the availability of codons. Specific to the VariFlex vectors containing the Q-tag, it is important to use an expression strain lacking functional β-galactosidase, such as the BL21-Gold(DE3)LacZ$^-$ strain.

The BL21-Gold(DE3)LacZ$^-$ expression strain is a spontaneous β-galactosidase deficient derivative of BL21-Gold(DE3), used for expression of proteins containing the Q-tag. This tag is based on a variant of α-complementation of β-galactosidase activity. The Q-tag contains the portion of the *lacZ* gene which encodes for the α portion of the β-galactosidase protein. Q-tagged proteins in cell lysates can be quantified in vitro after addition of the complementation fragment which restores β-galactosidase activity. Since BL21 strains are *lacZ*$^+$ (i.e. β-galactosidase positive), lysates from conventional BL21 strains can not be used in this assay.

Many genes that are expressed from the very strong T7 promoter can be toxic to the *E. coli* host cells. When using the BL21-Gold(DE3) strain as the primary host strain for cloning, some caution should be exercised because even low-level expression can result in accumulation of a toxic gene product.

In order to reduce basal activity of T7 RNA polymerase in the uninduced state, the BL21(DE3)pLysS strain carries a low-copy-number vector that carries an expression cassette from which the T7 lysozyme gene is expressed at low levels. T7 lysozyme binds to T7 RNA polymerase and inhibits transcription by this enzyme. On IPTG induction, overproduction of the T7 RNA polymerase renders low-level inhibition by T7 lysozyme virtually ineffective. In addition to inactivation of T7 RNA polymerase transcription, T7 lysozyme has a second function involving specific cleavage of the peptidoglycan layer of the *E. coli* outer wall. The inability of T7 lysozyme to pass through the bacterial inner membrane restricts the protein to the cytoplasm, allowing *E. coli* to tolerate expression of the protein. This second function of lysozyme confers the further advantage of allowing cell lysis under mild conditions. Cells expressing T7 lysozyme are subject to lysis under conditions that would normally only disrupt the inner membrane (e.g., freeze–thaw cycles or the addition of chloroform or a mild detergent such as 0.1% Triton® X-100) due to the action of the protein on the outer wall when the inner membrane is disrupted.

In cases in which target genes are too toxic to allow plasmids to be established in DE3 lysogens, T7 RNA polymerase can be delivered to the cell by infection with the bacteriophage CE6 by using the methods outlined in the *Lambda CE6 Induction Kit* Instruction Manual (Catalog #235200), which is compatible with the VariFlex expression vectors. By using the method employed by the Lambda CE6 induction kit, no T7 RNA polymerase is present in the cell until the desired time of induction. The bacteriophage CE6 expresses T7 RNA polymerase from the lambda p_L and p_I promoters and carries the *Sam7* lysis mutations. This bacteriophage will allow effective expression of target genes in BL21 cells and presumably other nonrestricting hosts that absorb lambda. The phage can be propagated in the LE392 host strain [e14- (McrA-) *hsdR514 supE44 supF58 lacYI*],[9] which suppresses the *Sam7* mutation and therefore allows lysis of infected cells.

BL21 expression strains addressing codon usage issues are also available. Efficient production of heterologous proteins in *E. coli* is frequently limited by the rarity of certain tRNAs that are abundant in the organisms from which the heterologous proteins are derived. Forced high-level expression of heterologous proteins can deplete the pool of rare tRNAs and stall translation, resulting in low protein yields. Availability of tRNAs allows high-level expression of many heterologous recombinant genes in BL21-CodonPlus cells that are poorly expressed in conventional BL21 strains. BL21-CodonPlus® strains are engineered to contain extra copies of genes that encode the tRNAs that most frequently limit translation of heterologous proteins in *E. coli*.

BL21-Gold (DE3) LacZ⁻ Genotype

Host strain	Genotype
BL21-Gold (DE3) LacZ⁻	*E. coli* B F^- *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA lacZ* Hte

- Perform a complete DNA digestion with the appropriate enzymes. Use *Nco* I and *Bam*H I for the pBEc vectors, carefully ensuring that the proper coding sequence of the insert is in frame with the C-terminal tag(s). If the insert to be cloned contains one or more internal *Nco* I or *BamH* I sites, PCR primers may be engineered to include restriction sites with overhangs compatible with *Nco* I (e.g., *Afl* III, *Bsp*H I, *Sty* I) or *Bam*H I (e.g., *Bgl* II, *Bcl* I, *Bst*Y I). If the insert contains only internal *Nco* I sites, clone within the *Nhe* I and *Bam*H I sites.

- Any of the sites in the MCS can be used for the pBEn vectors; however, ensure that the proper coding sequence of the insert is in frame with the N-terminal tag (see the MCS regions in Figure 2).

- Stratagene suggests dephosphorylation of the digested VariFlex protein expression vector with CIAP prior to ligating to the insert DNA. If more than one restriction enzyme is used, the background can be reduced further by electrophoresing the DNA on an agarose gel and gel purifying the desired vector band eliminating the small fragment excised from between the two restriction enzyme sites.

- After gel purification, resuspend in a volume of TE buffer (see *Preparation of Media and Reagents*) that will allow the concentration of the vector DNA to be the same as the concentration of the insert DNA (~0.1µg/µl).

LIGATING THE INSERT

For ligation, the ideal insert-to-vector ratio of DNA is variable; however, a reasonable starting point is 2:1 (insert-to-vector molar ratio), measured in available picomole ends. This is calculated as follows:

$$\text{Picomole ends / microgram of DNA} = \frac{2 \times 10^6}{\text{number of base pairs} \times 660}$$

1. Prepare three control and two experimental 10-µl ligation reactions by adding the following components to separate sterile 1.5-ml microcentrifuge tubes:

   Note *For blunt-end ligation, reduce the rATP to 0.5 mM and incubate the reactions overnight at 12–14°C.*

Ligation reaction components	Control			Experimental	
	1[a]	2[b]	3[c]	4[d]	5[d]
Prepared vector (0.1 µg/µl)	1.0 µl	1.0 µl	0.0 µl	1.0 µl	1.0 µl
Prepared insert (0.1 µg/µl)	0.0 µl	0.0 µl	1.0 µl	*X* µl	*X* µl
rATP [10 mM (pH 7.0)]	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
Ligase buffer (10×)[e]	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.0 µl	0.5 µl	0.5 µl	0.5 µl
Double-distilled (ddH_2O) to 10 µl	6.5 µl	7.0 µl	6.5 µl	*X* µl	*X* µl

[a] This control tests for the effectiveness of the digestion and the CIAP treatment. Expect a low number of transformant colonies if the digestion and CIAP treatment are effective.

[b] This control indicates whether the vector is cleaved completely or whether residual uncut vector remains. Expect an absence of transformant colonies if the digestion is complete.

[c] This control verifies that the insert is not contaminated with the original vector. Expect an absence of transformant colonies if the insert is pure.

[d] These experimental ligation reactions vary the insert-to-vector ratio. Expect a majority of the transformant colonies to represent recombinants.

[e] See *Preparation of Media and Reagents*.

2. Incubate the reactions for 2 hours at room temperature (22°C) or overnight at 4°C.

TRANSFORMING THE LIGATION REACTIONS

Following subcloning into a routine cloning host strain, positive transformants are then used to transform a protein expression strain such as BL21-Gold(DE3) or BL21-Gold(DE3)LacZ$^-$ competent cells.

Transformation Guidelines

It is important to store the competent cells at –80°C to prevent a loss of efficiency. For best results, please use the guidelines outlined in the following sections.

Storage Conditions

The competent cells are very sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. The competent cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep the competent cells on ice at all times. It is essential that the 14-ml BD Falcon polypropylene round-bottom tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use at least 100 µl of competent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of 14-ml BD Falcon Polypropylene Tubes

The use of 14-ml BD Falcon polypropylene round-bottom tubes when transforming into BL21 cells is imperative as the critical heat-pulsing period is calculated for the thickness and shape (i.e., the round bottom) of these tubes.

Quantity of DNA Added

Greatest efficiencies are observed when adding 1 µl of 0.1 ng/µl of DNA/100 µl of cells. A greater number of colonies will be obtained when plating up to 50 ng, although the overall efficiency may be lower.

Length of the Heat Pulse

Optimal transformation efficiencies are observed when transformation reactions are heat-pulsed for 20–25 seconds at 42°C. Transformation efficiencies decrease sharply when the duration of the heat pulse is <20 seconds or >25 seconds.

Transformation Protocol

1. Thaw the BL21-Gold(DE3) or BL21-Gold(DE3)LacZ$^-$ competent cells on ice.

   Note *Store the competent cells* ***on ice at all times*** *while aliquoting. It is essential that the 14-ml BD Falcon polypropylene tubes are placed on ice before the competent cells are thawed and that 100 µl of competent cells are aliquoted directly into each* ***prechilled*** *polypropylene tube. Do not pass the frozen competent cells through more than one freeze–thaw cycle.*

2. Gently mix the competent cells. Aliquot 100 µl of the competent cells into the appropriate number of prechilled 14-ml BD Falcon polypropylene tubes.

3. Add 1–50 ng of DNA to each transformation reaction and swirl gently. For the control transformation reaction, add 1 µl of the pUC18 control vector (100 pg) to a separate 100-µl aliquot of the competent cells and swirl gently.

4. Incubate the reactions on ice for 30 minutes.

5. Heat-pulse each transformation reaction in a 42°C water bath for 20 seconds. **The duration of the heat pulse is critical for optimal transformation efficiencies (see *Length of the Heat Pulse*).**

6. Incubate the reactions on ice for 2 minutes.

7. Add 0.9 ml of preheated SOC medium[§] to each transformation reaction and incubate the reactions at 37°C for 1 hour with shaking at 225–250 rpm.

8. Concentrate the cells transformed with the ligation reaction by centrifugation and plate the entire transformation reaction (using a sterile spreader) onto a single LB–ampicillin agar plate.[§,‖]

   To plate the cells transformed with the pUC18 control vector, first place a 195-µl pool of SOC medium on an LB-ampicillin agar plate. Add 25 µl of the control transformation reaction to the pool of SOC medium. Use a sterile spreader to spread the mixture.

9. Incubate the plates overnight at 37°C.

[§] See *Preparation of Media and Reagents*.

[‖] When spreading bacteria onto the plate, tilt and tap the spreader to remove the last drop of cells. If plating <100 µl of the transformation reaction, plate the cells in a 200-µl pool of SOC medium. If plating ≥100 µl, the cells can be spread directly onto the plates.

Expected Transformation Results

Host strain	Quantity of transformation plated	Expected colony number	Efficiency (cfu/µg of pUC18 DNA)
BL21-Gold(DE3)	25 µl	≥250	≥1 × 10^8
BL21-Gold(DE3)LacZ$^-$	25 µl	≥125	≥5 × 10^7

INDUCTION OF THE TARGET PROTEIN USING IPTG

The following induction protocol is a general guide for expression of genes under the control of IPTG-inducible promoters on an analytical scale (1-ml of induced culture). Most commonly, this protocol is used to analyze protein expression of individual transformants of BL21(DE3) host strains in combination with vectors containing T7 promoter constructs (e.g., pBE or pET vectors).

Note *The transformation procedure described above will produce varying numbers of colonies depending on the efficiency of transformation obtained using the expression vector. It is prudent to test more than one colony as colony-to-colony variations in protein expression are possible.*

1. Inoculate 1-ml aliquots of NZY broth containing 100 µg/ml of carbenicillin or ampicillin (see *Preparation of Media and Reagents*) with single colonies from the transformation. Shake at 220–250 rpm at 37°C overnight.

   Note *If the competent cells contain a pACYC-based vector (e.g., any BL21-CodonPlus strain or the BL21(DE3)pLysS strain), the overnight culture must include chloramphenicol at a final concentration of 50 µg/ml in addition to the carbenicillin/ampicillin required to maintain the pBE vector.*

2. Following overnight incubation, pipet 50 µl of each culture into fresh 1-ml aliquots of NZY broth containing no selection antibiotics. Incubate these cultures with shaking at 220–250 rpm at 30°C for 3 hours.

3. Pipet 100 µl of each of the cultures into clean microcentrifuge tubes and place the tubes on ice until needed for gel analysis. These will serve as the non-induced control samples.

4. To the rest of the culture in each tube add IPTG to a final concentration of 0.1–1 mM. Incubate with shaking at 220–250 rpm at 30°C for 1–3 hours.

   Note *These values for IPTG concentration and induction time are starting values only and may require optimization for the expression of different gene products.*

5. After the end of the induction period, place the cultures on ice.

AFFINITY PURIFICATION OF THE SBP-TAGGED PROTEIN

Preparing the Streptavidin Resin

Note *The volumes of resin and buffer given should be optimized to your experimental parameters.*

1. Centrifuge 50 µl of 50% streptavidin resin slurry (per 1 ml culture) at 1500 × *g* for 5 minutes to collect the resin. Discard the supernatant to remove the ethanol storage buffer. Resuspend the resin in 50–100 µl of streptavidin binding buffer (see *Preparation of Media and Reagents*). Repeat this wash step.

2. Collect the resin by centrifugation at 1500 × *g* for 5 minutes. Discard the supernatant and resuspend the resin in 25 µl of streptavidin binding buffer.

Purifying the SBP-tagged Protein Using Streptavidin Resin

1. Following induction by IPTG, pellet the cells by centrifugation at 2000 × *g* for 15 minutes. Discard the supernatant.

2. Resuspend the cells in 500 µl of streptavidin binding buffer and protease inhibitor(s).

   Note *If desired, lysozyme may be added to this mixture, and requires incubation on ice for 15 minutes.*

3. Lyse the cells by sonication.

4. Pellet the cell debris by centrifugation for 5 minutes at 12,000 × *g*. Retain the supernatant, which contains the expressed proteins.

5. For each 1 ml culture, add the supernatant to 50 µl of the prepared streptavidin resin (50% slurry). Rotate the tube at 4°C for 30 minutes to allow the tagged proteins to bind to the resin.

6. Collect the resin by centrifugation at 1500 × g for 5 minutes. Remove and freeze the supernatant for possible analysis later. Resuspend the resin in 1 ml of streptavidin binding buffer by rotating the tube at 4°C for 5 minutes.

7. Repeat step 6 twice, for a total of three washes.

8. Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant. Add 100 µl of streptavidin low salt elution buffer[§] to the resin. For cases when the protein fails to elute completely from the resin, use the high salt elution buffer[§].

9. Rotate the tube at 4°C for 30 minutes to elute the protein.

10. Collect the resin by centrifugation at 1500 × g for 5 minutes. Carefully transfer the supernatant to a fresh tube (the supernatant contains the eluted protein).

[§] See *Preparation of Media and Reagents*.

PROTEIN QUANTIFICATION USING THE Q-TAG

Q-tagged proteins must be expressed in the BL21-Gold(DE3)LacZ$^-$ host strain to minimize the β-galactosidase activity contributed by the expression strain. Since BL21 strains are *lacZ*$^+$ (i.e. β-galactosidase positive), lysates from conventional BL21 strains can not be used in this assay.

1. Following induction by IPTG, pellet the cells by centrifugation at 2000 × *g* for 15 minutes. Discard the supernatant.

2. For a 1 ml cell culture, resuspend the cells in 500 µl of the cell lysis buffer provided and protease inhibitor(s).

   Note *If desired, lysozyme may be added to this mixture, and requires incubation on ice for 15 minutes.*

3. Lyse the cells by sonication.

4. Pellet the cell debris by centrifugation for 5 minutes at 12,000 × *g*. Retain the supernatant, which contains the expressed proteins.

5. Dilute the supernatant in cell lysis buffer. Transfer 30 µl of the diluted cell lysate into a fresh tube.

   Note *Since expression in the pET system usually results in very high yields, the lysates need to be diluted to avoid saturation of the signal. Typically a 1:1000 dilution of the lysate is required.*

6. Transfer 1 µl of the ED control and 29 µl of cell lysis buffer into a separate tube.

7. Add 20 µl of the EA reagent to each of the tubes containing 30 µl of the diluted cell lysate or ED control. Incubate the tubes for 15 minutes at room temperature.

8. Add 40 µl of freshly prepared substrate solution (see *Preparation of Media and Reagents*) to each tube.

9. Incubate the tubes at room temperature for 30 minutes.

10. Measure chemiluminescence either in a single-tube format or in a plate reader according to manufacturer's recommendations.

Notes *The ED control results will vary due to differences in instrument settings, and is intended to confirm β-galactosidase complementation and to reassure that the assay components are functional.*

The BL21-Gold(DE3)LacZ– expression strain, despite the β-galactosidase being catalytically inactive, can still perform α-complementation. Lysates from this expression strain will result in detectable β-galactosidase activity in the in vitro complementation assay, although the level of β-galactosidase activity is approximately 100-fold lower as compared to the wild-type BL21 strain.

REMOVING THE TAGS WITH THROMBIN

Ideal digestion conditions will vary between proteins and should be optimized for each fusion protein. Stratagene recommends starting with a 1:500 thrombin-to-fusion protein ratio and analyzing the reaction products at various time points from several minutes to 24 hours following the addition of thrombin. A lower thrombin-to-target ratio (e.g., 1:50) may be used to decrease long reaction times. Dialyze or dilute the tagged fusion protein into thrombin cleavage buffer (see *Preparation of Media and Reagents*). Add the thrombin to the reaction tube and incubate at room temperature until cleavage is complete. Determine the efficiency of proteolytic removal of the tag(s) by SDS–PAGE analysis.

Note *Thrombin may be inactivated by the addition of protease inhibitor(s).*

TROUBLESHOOTING

Observations	Suggestions
Vector instability	Unstable DNA sequence. Prior to induction of cultures, assay for colony formation by plating cells on an LB plate and an LB-ampicillin plate. If the vector contains unstable DNA sequence one should observe colony formation on the LB plate, and reduced colony formation on the LB-ampicillin plate.
	Overexpression of toxic proteins. Prior to induction of cultures, assay for colony formation by plating cells on an LB-ampicillin plate and an LB-ampicillin plate containing IPTG. If the insert codes for a protein that is toxic to the cells, overexpression of the toxic protein should result in reduced colony formation on an LB-ampicillin plate containing IPTG as compared to cells plated on the LB-ampicillin plate.
	More tightly controlled induction may be achieved by performing induction by infecting BL21 cells with the bacteriophage CE6, however use of other BL21 cells are not compatible with the B-gal quantitation assay.
Problems associated with induction time	Depends on the physicochemical characteristics of the protein and toxicity of the protein to *E. coli*. In certain cases, accumulation of target protein may kill cells at saturation while allowing normal growth in logarithmically growing cultures, while in other cases target protein may continue to accumulate in cells well beyond the recommended 3-hour induction period. To determine the optimal induction period, a time course may be carried out during which a small portion of the culture is analyzed by SDS–PAGE at various times following induction.
Inclusion bodies	Improper folding in *E. coli* and/or bacterial aggregation due to the physical properties of the protein. In some cases, protein may form insoluble inclusion bodies at 37°C. In many cases, this protein may be soluble and active if the induction is carried out at 30°C.
	Use the expression vectors containing the SET tags, which may reduce insoluble inclusion body formation.
Precipitation of fusion protein observed in elution fractions	Insufficient ionic strength in the elution buffer and/or the pH of the elution buffer is inappropriate for the pH of the fusion protein. Optimize the buffer system to correct the ionic strength in the elution buffer or correct the pH of the elution buffer affecting the pH of the fusion protein.
Contaminating proteins coeluting with fusion protein	The use of nonionic detergents, such as NP-40 and Triton X-100, at 0.1% may be effective in the elimination of contaminating proteins.
Protein fails to elute completely from the resin	Protein precipitated on the resin. Increase NaCl concentration of the elution buffer. Add detergent to the elution buffer.
	Biotin concentration in the elution buffer too low. Ensure that biotin concentration in streptavidin elution buffer is 2 mM.
Incomplete proteolytic cleavage	The efficiency of proteolytic removal of the tags by thrombin will vary from protein to protein, and in some cases, the conformation of the protein may inhibit accessibility of the thrombin cleavage target site for the enzyme. Longer incubation times or higher concentrations of protease may help.
	Positioning of the tag at the opposite terminus of the protein of interest by recloning the insert into the appropriate protein expression vector may increase accessibility of the target site.

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Cool to 55°C.
Add appropriate antibiotic.
Pour into petri dishes (~25 ml/100-mm plate)

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB–Ampicillin Broth (per Liter)

1 liter of LB broth, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin

LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Carbenicillin Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 10 ml of 10-mg/ml-filter-sterilized carbenicillin

SOB Medium (per Liter)

20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Autoclave
Add 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M $MgSO_4$ prior to use
Filter sterilize

SOC Medium (per 100 ml)

SOB medium
Add 1 ml of a 2 M filter-sterilized glucose solution or 2 ml of 20% (w/v) glucose prior to use
Filter sterilize

NZY Broth (per Liter)

5 g of NaCl
2 g of $MgSO_4 \cdot 7H_2O$
5 g of yeast extract
10 g of NZ amine (casein hydrolysate)
Add deionized H_2O to a final volume of 1 liter
Adjust the pH to 7.5 with NaOH
Autoclave

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction*

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

Streptavidin Binding Buffer 10 mM Tris HCl, pH 8.0 150 mM NaCl	**Low-Salt Streptavidin Elution Buffer** 10 mM Tris HCl, pH 8.0 150 mM NaCl 2 mM biotin
	High-Salt Streptavidin Elution Buffer 10 mM Tris HCl, pH 8.0 1 M NaCl 2 mM biotin
Substrate Solution Combine the following reagents (provided in the Q-tag detection kit) and gently mix by inversion: 1 part CL substrate 5 parts CL enhancer 19 parts CL substrate diluent Once prepared, stable for 24 hours at 2–8°C	**Thrombin Cleavage Buffer** 20 mM Tris-HCl (pH 8.4) 150 mM NaCl 2.5 mM $CaCl_2$

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ENDNOTES

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